THE AMINO ACID COMPOSITION OF THE MAJOR HEMOGLOBIN COMPONENT OF CLETHRIONOMYS RUTILUS DAWSONI

A

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RUTILUS DAWSONI

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ABSTRACT

The amino acid composition was determined for the tryptic peptides of the major component of the hemoglobin of Clethrionomys rutilus dawsoni by peptide mapping and amino acid analysis of the eluted peptides.

Relative to the 287 amino acids of human hemoglobin, 191 amino acids of Clethrionomys Hb-f are ascribed to specific tryptic peptides. When compared to the compostion of the hemoglobins of two other Microtines, the 24 peptides from the alpha and beta chains of Clethrionomys Hb-f differed in 7 amino acids with Microtus pennsylvanicus Hb and in 8 amino acids with Microtus abbreviatus Hb-f.

On the basis of homology with known human hemoglobin A, a sequence was inferred for Clethrionomys Hb-f. This proposed sequence fits the description of hemoglobin as composed of folded helical globin molecules with exclusively non-polar interiors.

PREFACE

It is the purpose of this research to describe the primary structure of the major hemoglobin component of *Clethrionomys rutilus dawsoni*. Techniques were employed which permit a description of the primary structure in terms of amino acid composition.

Gratitude is expressed to Dr. Charles Genaux for his guidance, encouragement and prodding and to Dr. Peter Morrison for financial assistance and for permission to use Institute of Arctic Biology facilities for much of this research.

Thanks must also be expressed to my parents, husband and members of the Chemistry Department for their faith in me.

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INTRODUCTION

Explanation of the interrelationships between the structure and biological function of molecules is the main theme of biochemistry. The classical example of such a relationship between structure and function resulted from investigations of hemoglobin S (Hb S) which is found to occur in sickle cell anemia. Studies have shown sickle cell anemia to be an inherited molecular disease caused by the substitution of only one amino acid residue, valine, for a glutamic acid residue of the normal human hemoglobin molecule. (Pauling et al., 1949; Ingram, 1958). This substitution apparently results in the low solubility of deoxygenated hemoglobin S which causes a distortion of the red blood cell.

Hemoglobin has since been the subject of numerous investigations and may be considered to be one of the best characterized of all proteins. The structure and function of many abnormal human hemoglobins as well as those of the hemoglobins of many other species of animals have been investigated and compared (Schroder, 1963; Lush, 1966). Species differences have been found in such properties as crystal form, solubility, electrophoretic rates and patterns, affinity for oxygen and absorption spectra. These differences are entirely due to differences in the primary structure of the globin, i.e. the number and sequence of amino acids in the globin, since the same ferroprotoporphyrin is present in all vertebrate and many invertebrate hemoglobins. Therefore study of the composition of the globin is important in any attempt to understand the behavior of the hemoglobins.

Knowledge of the primary structure of hemoglobin, taken in conjunction with independent evidence of its conformation, i.e. its characteristic folded structure, has contributed much to an understanding of the function of hemoglobin. For example, on the basis of his X-ray crystallographic studies M.F. Perutz has discussed the total exclusion of polar residues from the interior of hemoglobins as well as the correspondence between the amino acid sequence and the corners or non-helical regions of the chains (Perutz, 1965).

The observed three dimensional conformation assumed by a protein appears to be determined solely by its primary structure; additional data on the primary structure may enable one, eventually, to predict the three dimensional structure of a protein from a knowledge of its sequence (Schroeder, 1968).

Several techniques have been especially useful in the study of the structure of hemoglobin. While early techniques gave only general indications as to its physical-chemical nature, later techniques have provided more specific information relevant to the primary structure of hemoglobin.

Electrophoresis of Proteins

During electrophoresis at a specific pH each protein demonstrates a characteristic mobility in the electric field due to its charge at that pH. Electrophoresis of human hemoglobin S and normal human hemoglobin A shows them to have different electrophoretic mobilities, and provides the first clue as to the nature of the difference between the two hemoglobins (Pauling et al., 1949). Electrophoresis has found wide usage since Pauling's initial work. Hemoglobin mobilities have been measured to characterize them, to type them for genetic studies and to screen them for detection of abnormal hemoglobins. Care must be used in interpreting and comparing the electrophoretic patterns of hemoglobins. Two electrophoretic components do not necessarily correspond to the existence of two chemically different hemoglobin components (Riggs, 1965a; Bonaventura and Riggs, 1967). It should be noted too that screening for mutant hemoglobins exclusively with electrophoretic means will not detect mutant hemoglobins with replacements involving neutral amino acids. Gel electrophoresis as described by Jovin et al. (1964) has been sucessfully used in the preparation and purification of hemoglobins for study.

Peptide Mapping

More specific information about hemoglobin became available because of the method of peptide mapping devised by Ingram which involves subdivision of the globin and a separation of the resulting peptides (Ingram, 1956). The subdivision of the hemoglobin is effected most often by the enzyme trypsin which specifically cleaves the peptide bonds associated with the carboxyl groups of lysine and arginine. Because of the nature of the selective cleavage by trypsin, each tryptic peptide is expected to contain only one lysine or arginine residue, and that residue is expected to occupy the carboxyl-terminal position in the peptide. Peptide mapping or "fingerprinting" is the method by which the tryptic peptides are separated into a two dimensional array on paper. By virtue of their characteristic charged groups, the peptides can be caused to migrate in an electrophoretic field. According to their characteristic hydrophobic or hydrophilic groups, the peptides can then be partitioned selectively by chromatography in the second dimension. The peptides are located by staining the paper with ninhydrin.

Ingram observed that the peptide maps of human hemoglobins A and S differed in the location of only one peptide (Ingram, 1958). He eluted the two peptides from their maps and detected a single difference in amino acid composition of the peptides. Bagliono (1961) described an improved method of peptide mapping and also made use of stains for specific amino acids to aid in identifying the peptides. Dawson et al. (1959) reviewed the stains for specific amino acids.

Since the initial work by Ingram, peptide mapping has been extensively used in attempts to compare hemoglobin structures. For example, C.W. Foreman (1964) compared the peptide maps of the hemoglobins of six species of Peromyscus and also presented a composite drawing of maps of Clethrionomys gapperi and Microtus pennsylvanicus which indicated differences involving at least 13 peptides.

Hutton et al. (1962) separated the alpha and beta chains of hemoglobins of several strains of mice and compared their peptide maps. Popp (1962) did essentially the same thing with several other strains of mice. Popp was careful to point out however, that while he could find no differences between the alpha chains of the different strains by this procedure of peptide mapping, a failure to find differences does not mean that the chains are identical.

Amino Acid Analysis of Proteins and Peptides

Quantitative determination of the amino acid composition of proteins became possible when in 1948 Moore and Stein described a chromatographic method that requires only a few milligrams for a complete analysis (Stein and Moore, 1948;

Moore and Stein, 1948 and 1949). Following acid hydrolysis of the protein, the constituent amino acids are separated and identified. The method was developed until Spackman, Stein and Moore (1958) described an automatic amino acid analyzer which could perform a complete analysis in less than 24 hours. This method did not accurately detect the replacement of one or two amino acid residues in a molecule the size of hemoglobin and so did not indicate the nature of the difference between the human hemoglobins A and S.

Amino acid analysis of the separated tryptic peptides allows improved precision and improved ability to discriminate between the amino acid composition of hemoglobins. Therefore the procedure of cutting out the peptide spots, eluting them and performing an amino acid analysis has become an important addition to the peptide mapping procedure. Clegg and his colleagues (1966) compared peptide maps of variant human hemoglobins, cut out homologous peptides with different locations and analyzed them. Popp (1965) and Kilmartin and Clegg (1967) have eluted all peptide spots from their peptide maps and analyzed them. Popp reported in 1965 that "peptide analyses by fingerprinting alone, especially under one set of conditions, do not detect all amino acid replacements." The results of Genaux (1969) and of this research support Popp's conclusion.

Gross comparison of peptide maps generally is of limited value in structural analysis. An overlay of the peptide maps gives only an indication of the degree of similarity between the globins. A comparison of the peptide map of an unknown hemoglobin with the map of a hemoglobin whose tryptic peptides have been mapped and identified gives only an indication of the identity of the unknown peptides. However, peptide mapping combined with amino acid analysis of the tryptic peptides affords an accurate comparison of structure in terms of the amino acid composition. A similiar method used by several researchers has been the separation of the tryptic peptides by column chromatography followed by amino acid analysis of the peptides (Rifkin et al, 1966; Hilse and Popp, 1968).

Although amino acid analysis of tryptic peptides does not allow one to specify the amino acid sequence, it is possible for one to infer a sequence on the basis of homology with a hemoglobin of a known sequence.

Sequence Determination

To completely define the primary structure of hemoglobin ultimately requires determination of the amino acid sequence. The first complete sequence determination of a protein was reported by Sanger (1956) and was based on a method of determining the N-terminal amino acids by means of the reagent fluorodinitrobenzene. This work of Sanger was a breakthrough in the field of chemical structural analysis of proteins. In the early 1960's the complete primary structure of human hemoglobin A was reported (Braunitzer et al., 1961; Hill and Konigsberg, 1962; and Schroeder et al., 1963).

The Edman method or the PTH procedure has become the most common method of sequence determination. Described by Edman (1950) it involves the removal of one residue at a time sequentially from the N-terminal of a peptide. A "protein sequenator", an automatic apparatus which carries out about 15 cycles of degradation in 24 hours has been described by Edman and Begg (1967).

Analysis of the structure of a protein may currently be pursued through sucessive stages of difficulty according to the point of view of the researcher and according to the techniques at his disposial.

MATERIALS AND METHODS

CLETHRIONOMYS

Animals used in this work were of the species *Clethrionomys rutilus dawsoni* (red-backed voles), the family Cricetidae, sub-family Microtinae of the order Rodentia. They were all laboratory bred in the animal colony at the Institute of Arctic Biology. The colony of *Clethrionomys* used in this work was established from wild stock obtained in the geographic area surrounding Fairbanks, Alaska, and are designated by the species code 5822AAI.

PREPARATION OF HEMOGLOBIN AND GLOBIN

Blood, obtained from *Clethrionomys* by decapitation, was collected in small plastic vessels with heparin and in heparinized capillary tubes. Up to 0.5 ml of red blood cells could be obtained in this way depending upon the size of the animal. The blood was immediately diluted with 0.85% saline, was centrifuged in the refrigerated high speed centrifuge Servall RC-2 at 3000 rpm for several minutes, and was subsequently washed three times with saline solution. Lysis of the cells was effected by the addition of one to four volumes of water and freezing of the hemolysates. After the hemoglobin solution was thawed and centrifuged at 9000 rpm at 0^{o} C for 30 minutes, the supernate containing hemoglobin was drawn off from the cell debris and stored in the refrigerator.

Gel Electrophoresis

Two kinds of acrylamide gel electrophoresis were performed with the hemoglobin: analytical gel electrophoresis and a modified preparative disc gel electrophoresis. The analytical gel electrophoresis was performed on a vertical gel sheet with appartus designed by Raymond (1962).[1] This procedure provided initial evidence for the two components of Clethrionomys hemoglobin as well as for the general purity of the isolated hemoglobin, i.e. absence of other non-heme proteins.

The following conditions were used for the electrophoresis with the Raymond apparatus. A stock solution for pH 8.6 buffer was prepared according to Boyer et al (1963): 54.5 g TRIS (tris-hydroxylmethylaminomethane, Matheson, Coleman and Bell), 15.5 g boric acid, and 3.4 g Na_2EDTA (ethylenediaminetetraacetic acid, disodium salt, Sigma) were dissolved in water and made up to a volume of one liter. For use as an electrolyte in the Raymond apparatus 200 ml of the stock buffer solution were diluted to two liters. A stock solution of acrylamide was prepared according to Peterson (1963): 70 g acrylamide (Eastman) 2.0 g BIS (N,N' methylenebisacrylamide, Eastman),

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[1] Commercial model available from E-C Apparatus Corp, 222 South 40th Street, University City, Philadelphia, Pa., 19104; Technical Bulletin 141

1.4 ml TEMED (N,N,N',N', tetramethylethylenediamine, Eastman) and 110 ml of the stock solution of pH 8.6 buffer were made up to a total volume of one liter.

For preparation of the gel sheet 300 mg of ammonium persulfate (Mallinckrodt) were dissolved with vigorous mixing in 200 ml of acrylamide stock solution. The solution was immediately poured into the assembled apparatus where the gel set in about five minutes. The apparatus rested horizontally with the slot-former in place while the gel solidified.

Following solidification, the top of the gel was trimmed and the slot-former was removed. Eight different samples could be run at the same time in the eight slots provided. Sucrose was added to the hemoglobin samples to make a solution of about 20% in sucrose and about 5% in hemoglobin. The dense solution could be layered in the slots with no diffusion of hemoglobin. A micrometer pipette with thin plastic tubing was used to load about 10 λ per slot. A voltage which ranged from 100 to 600 v was applied across the top and bottom of the gel in order to maintain a current of about 65 ma. The hemoglobin migrated toward the positive electrode when the pH of the buffer at both electrodes was 8.6.

Following electrophoresis the gel sheet was removed and stained approximately twenty minutes in a 0.1% solution of amido black (10 B electrophoretic grade, Harleco) in 7% acetic acid. The sheet was then destained in portions of fresh 7% acetic acid.

Preparative gel electrophoresis, a method of separating and isolating multiple proteins from solution according to differences in electric charge, was applied to the separation of the two hemoglobins of Clethrionomys using the techniques of Jovin et al (1964) and Genaux (1969) together with the commercial Canalco Prep-Disc Electrophoresis Apparatus.[2] The largest glass column PD 2/320 was used with a short annular column of separating gel approximately 3 cm tall.

The following conditions were used for the modified preparative gel electrophoresis. To prepare the upper buffer stock solution, 28.8 g glycine (Cal Biochem) and 6.0 g TRIS were dissolved in water and made up to a volume of one liter. For use, one volume of the stock was diluted to ten volumes. This buffer, electrolyte for the cathode, had a pH of 8.3. The lower buffer, electrolyte for the anode, was prepared by adding 60 ml of N HCl to about 900 ml of water and adding solid TRIS to bring the final one liter of solution to a pH of 8.1. The gel stock solution was prepared by mixing 24.0 ml of N HCl, 18.15 g TRIS and 0.23 ml TEMED, 30 g acrylamide, and 0.8 g BIS in enough water to bring the final volume to 200 ml.

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[2]Canal Industrial Corporation, 5635 Fisher Lane, Rockville, Md. 20852.

In preparation for electrophoresis, the stock gel solution was mixed 1:1 with fresh ammonium persulfate solution (70 mg per 50 ml water). After gentle but thorough mixing, 7.5 ml of the mixture were poured into the apparatus to which "Saran Wrap" had been attached securely over the bottom. (The bottom of the center elution tube was covered with a small square of tape.) Immediately after the gel was poured a small volume of water was layered on the top of the gel to give a flat gel surface. Although the gel appeared to solidify in about 10 min, it was allowed to stand overnight at room temperature. Before electrophoresis the "Saran Wrap" and cellophane tape were removed, and cellulose (dialyzer tubing, Thomas) was placed over the gel and securely attached.

About 0.9 ml of hemoglobin solution, 20% in sucrose, was layered on top of the gel, and a voltage ranging from 400 v to 900 v was applied between the anode and cathode to maintain the current between 5 ma and 10 ma. As the hemoglobin passed through the gel and then into the space between the gel and the cellulose, osmotic dilution forced the eluate upwards through the elution tube into a receiving vessel. A separation of a fast band (major hemoglobin component) and of a slow band (minor hemoglobin component) was effected as the <u>Clethrionomys</u> hemoglobin passed through the gel. After the major component had entered the elution tube and while the minor component was still in the gel, the apparatus was dismantled, the cellulose was removed, and the hemoglobin in the tube was collected. From 0.5 ml to 0.8ml of the major hemoglobin component was collected per run. Although the slow band could be collected by the same method it was not present in sufficient quantity to work with. One gel generally was used for the separation of three separate samples.

In the procedure described by Jovin et al. (1964) the cellulose membrane was not used over the bottom of the preparative gel. Instead, the lower buffer was passed through a small space, set between the gel and the bottom of the flow chamber, and thence up the elution tube at a rate of about 1 ml/min. The components which were obtained much diluted were then layered on a concentration gel with the cellulose membrane as described by Genaux (1969), which also represented a modification of the method of Jovin et al. (1964). The modified preparative method as employed in my work combined the two methods and necessitated only one operation for the isolation of one hemoglobin component in good concentration.

Globin Precipitation

The hemoglobin was converted to globin following a procedure described by Jovin et al. (1964) and Clegg et al. (1966). The hemoglobin solution was added drop by drop while stirring to a 15 to 20 volume excess of 2% by volume concentrated HCl in acetone (Baker Analyzed) kept at -20°C or below in a dry-ice cooled methanol bath. The precipitate was washed once with acid acetone, twice with acetone with a trace of 2-mercaptoethanol (Aldrich) and twice with ether (Baker Analyzed). All reagents were cooled initially to about -20°C. The ether washed precipitate was then air dried.

<u>Conversion of Globin into S-(Beta-Aminoethyl) Derivatives</u>

The derivatization of the globin was performed by the methods of Clegg et al (1966) and Jones (1964). About 48 g of urea (Baker Analyzed) were put into a beaker with about 20 ml water and 10 g AG 501X8 resin (Cal Biochem, 20-50 mesh). Water was added and the solution heated in a hot water bath until the urea dissolved. The volume (including the resin) was brought to 100 ml. (The conductivity of the resultant solution was about 10 μmho.) Following filtration, which removed the resin from the mixture, 12.11 g TRIS and 15 mg EDTA were added to the filtrate, and the pH was adjusted from about 11 to 9 with concentrated HCl. The resultant solution was M in TRIS and 8 M in urea.

Aliquots of about 7.5 ml of deionized solution were used to dissolve 10 to 20 mg of globin in small vials. The globin solutions were stirred with magnetic bars in a covered beaker flushed with prepurified nitrogen gas (Matheson). After 30 min, which was sufficient for the globin to dissolve, 0.1 ml of 2-mercaptoethanol were added to each vial and stirring was continued 45 to 60 min. Since samples should not react longer than 30 min with ethyleneimine or side products will result, each vial was treated separately in the following manner: Each sample was treated with 0.1 ml of ethyleneimine and the reaction mixture stirred under nitrogen for 30 min. The pH was adjusted to about 3 with concentrated HCl, and the solution was layered on a column of G-25 Sephadex (Pharmacia), 2X25 cm, equilibrated with 0.5% formic acid. The protein was eluted with 0.5% formic acid at a rate of 1 to 2 ml/min. Fractions of 3 ml each were collected with the GME[3] fractionator, and a record of the ultraviolet absorption was obtained with an Esterline Angus recorder (Graphic Ammeter). Clean separation of the protein and urea-HCl solution was indicated both by the recorded U.V. absorption and by tests for chloride turbidity with 1 M $AgNO_3$. The protein in the 0.5% formic acid was lyophilized.

Trypsin Digestion

About 10 mg of globin were added to a small 10 ml vial containing 3 ml of freshly prepared 1% ammonium bicarbonate (Baker Analyzed) solution, and the mixture was stirred under nitrogen for about 30 min with a magnetic bar. A 0.1 ml aliquot of fresh trypsin solution (Worthington trypsin, bovine pancreas, TRL 100S, 3Xcrystalized, salt free, lyophilized, sterile; 1 mg/ml 0.001N HCl) was added to the vial, and the reaction was allowed to proceed up to 4 hours. Due to the heat of the "Mag-Mix" the temperature of the reaction vessel ranged from 28° to 33°C. During the

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[3]Gilson Medical Electronics, Middleton, Wisconsin.

digestion the pH of the solution changed from 8 to 8.7. The reaction was stopped by placing the vials in the freezer. The digested protein material was centrifuged at 7000 rpm for 30 min, the supernate was drawn off, and the insoluble material was washed twice with water. The two washings were added to the supernate, presumed to be the completely digested material, which was then lyophilized or repeatedly evaporated in the vaccuum desicator to insure removal of volatile salts.

Chymotrypsin Digestion

In order to recover any insoluble tryptic peptides, the insoluble material from the tryptic digest was treated with chymotrypsin which splits the peptide most rapidly at those bonds associated with the carboxyl groups of tryptophan, tryosine, phenylalanine and leucine. The washed, insoluble material from the tryptic digest of hemoglobin was transferred to small vials with portions of 3 ml of fresh 1% NH_4HCO_3 solution. The method of digestion with chymotrypsin (Worthington alpha-chymotrypsin, CD 100S, lyophilized, sterile; 0.1 ml of 1 mg/ml 0.001N HCl) was the same as described above for digestion with trypsin.

PEPTIDE MAPPING

The solute recovered by evaporation from the soluble fraction of the tryptic digest contained peptide fragments of from 1 to 30 amino acids in length as a result of the action of the trypsin which cleaves the peptide bonds associated with the carboxyl groups of lysine or arginine. These peptides can be separated by a two dimensional "mapping," i.e., consecutive electrophoresis and chromatography on paper. Methods of Ingram (1958) and the modifications of Clegg et al. (1966) and Genaux (1969) were used in the laboratory.

High Voltage Electrophoresis

The buffer, pH 6.4, for the high voltage electrophoresis on paper consists of pyridine (Mallenkrodt), acetic acid, and water in ratios by volume of 500:20:4500 respectively (Michl, 1951, cited by Ingram, 1958).

Sheets of Whatman 3MM chromatography paper 46X67 cm were used for the mapping of the tryptic digests. The paper was cut so that the majority of the peptides moved along the "machine direction" of the paper toward the cathode, thus giving a better separation than in the opposite direction. The papers were cut and marked as shown in Fig. 1. Rectangles were cut out of each end to leave wicks of 1 X 1½ inch and 5 X 1½ inch at the bottom or anode end and a single wick 1 x 3½ inch at the top or cathode end. A single wick 5 X 5 inches was cut from another paper and marked with a line 3½ inches from one end. The origin, at which the sample was applied, was located 3 inches from the right edge and 5 inches from the bottom. The penciled lines at the right edge were used as guides in folding the paper for descending chromatography. Two papers were

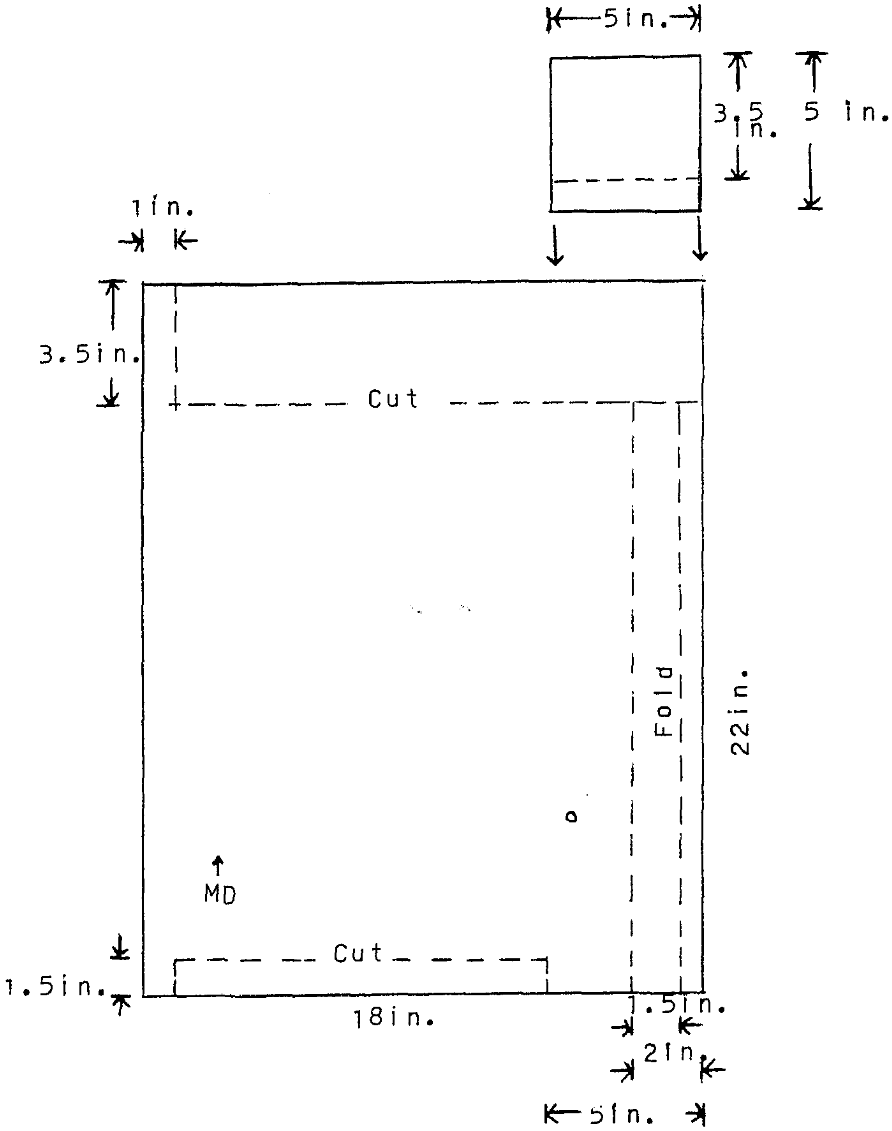

Figure 1. Preparation of Chromatography Paper for Peptide Mapping.

saturated with buffer and carefully arranged on the lucite support rack as shown in Fig. 2. Before the papers were clamped in place with the upper spiked retaining bar, the piece of 5X5 in 3MM paper saturated with buffer was interposed between the two papers at the upper right end so that a wick of 5X3½ in. was produced. The upper left wick on the underlying paper was cut off halfway down. Siphoning of buffer down the paper was prevented by the above two measures. The papers were arranged on the rack so that the two papers were not in contact with each other below the spiked retaining bar. Before the lowest bar was clamped into place, a total of 50 to 100 microliters of proteolysate solution (about 10 mg in 200 to 400 microliters of water) were applied at the origin of each paper in several applications.

A lucite tank[4] which can contain a total volume of 96 liters was used for the high voltage electrophoresis. Four to 6 liters of buffer covered the floor of the tank, immersing only the anode and the lower wicks of the papers on the rack. The cathode and the upper wicks of the papers were immersed in approximately 4 liters of buffer contained in separate elevated compartments between which electrical contact was made by a sandwich of 10 to 12 pieces of 3MM paper 7X18 in. The rest of the tank was filled with Chevron

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[4]Tank and support rack were constructed by Mr. Otto Hiller, P.O. Box 1294, Madison, Wis.

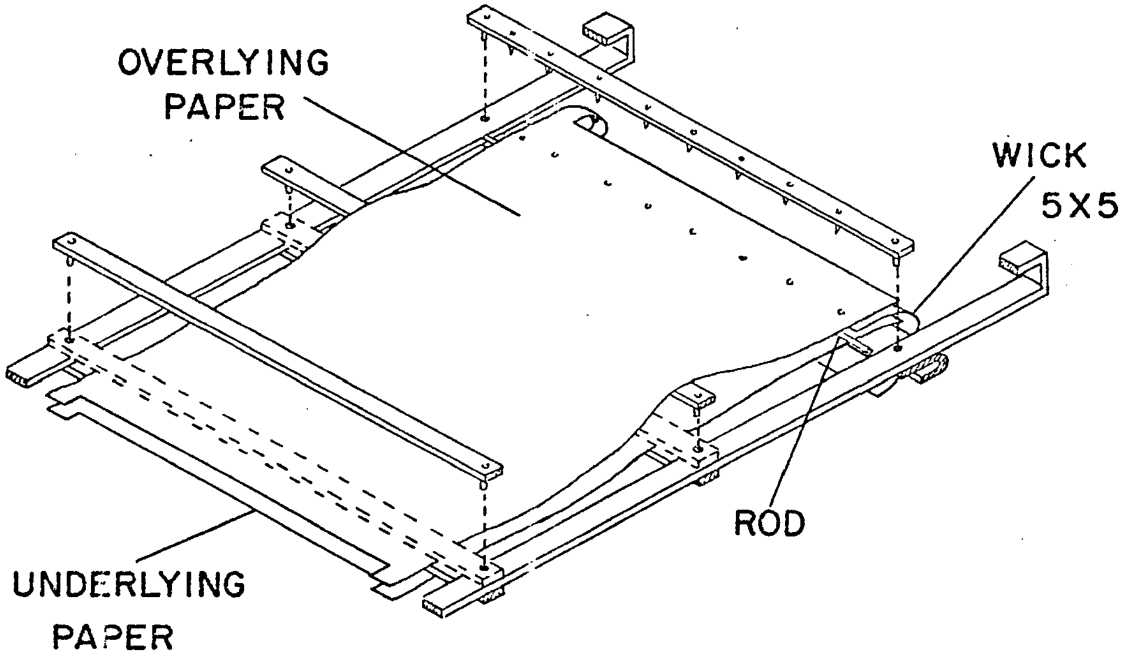

Figure 2. Arrangement of Chromatography Paper for Electrophoresis.

Stoddard Solvent, a petroleum fraction purchased from the local distributor for Standard Oil of California. This solvent was used in place of Varsol, which is cited in the literature, as the dielectric and cooling medium in high voltage electrophoresis. Cooling was produced by pumping ice water through a stainless steel coil. During an electrophoresis the temperature was maintained at about 20°C. Electrophoresis was carried out from 2½ to 4 hours at 1500 v and current changing from 100 to 150 ma.[5]

Descending Chromatography

After electrophoresis, the papers were dried, marked for identification, folded along the penciled lines previously described and hung in the Chromatocab (Research Specialties Co.) for descending chromatography. The "cab" can accommodate four troughs for the chromatography of eight papers. The cab was closed and the atmosphere saturated for 6 to 12 hours with a developer of 1-butanol: acetic acid: pyridine: and water in ratios of 300:60:200:240 by volume (Waley and Watson, 1953, cited in Clegg et al, 1966). After equilibration about 200 ml of developer was poured into each trough and the chromatography was begun. It took about 12 hours for the solvent to reach the bottom of the paper. The papers were developed for 12

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[5]The D.C. power supply, rated at 5000 v and 200 ma was manufactured by Canadian Research Institute, Don Mills, Ontario, Canada.

to 20 hours and were allowed to dry at room temperature.

Ninhydrin (Pierce Chemical Co.) in acetone was used to locate the position of the peptide spots. One map of each pair was dipped in a 0.2% solution of ninhydrin in acetone while the other was dipped in a 0.02% solution. For some maps, 5% by volume of the high voltage electrophoresis buffer was included in the ninhydrin solutions with the intention of sharpening the definition of the spots (Clegg et al, 1966). Since this seemed to have no effect and since there was the chance of spreading the spots (Toennies et al, 1951) this procedure was discontinued. After they were dipped, the papers were placed in a chromatography oven (New Brunswick Scientific Co.) constructed to avoid ignition of the acetone vapors. The oven was heated at 60°C for two 5 min periods which were separated by brief ventilation of the oven. Within 20 min the blue spots of ninhydrin-positive peptides became visible. They reached optimal definition after about 12 hours and were marked for identification. The spots were outlined with pencil and assigned an identifying number.

Elution of the Peptides for Acid Hydrolysis

The spots were cut from the maps with careful handling with forceps to avoid contamination from fingers. The excised spots were provided with small pointed tips on one side to which elution could be directed. The opposite side was cut off square to fit against the square end of a strip of 3MM paper retained between two microscope slides. The peptide spots were eluted with dilute HCl (fresh reagent concentrated HCl, 12N and water in ratios 1:1 by volume); in an arrangement derived from that described by Sanger and Tuppy (1951). The arrangement as shown in Fig. 3 provided a gentle siphoning action. Approximately 100 microliters of eluate were collected in capillary tubes (Scientific Products diSPo Micro Pipets). When the flow rate was faster than 100 microliters in 15 min or if the spot was particularily large, up to 300 microliters were collected to insure complete elution of the peptide from the paper. The filled capillary tubes were carefully sealed in an oxygen-gas flame to exclude as much air as possible, were labeled and then were heated at 105^{o}C for 24 hours. The resulting hydrolysates were washed into a 3 ml test tube and evaporated to dryness in a vacuum desiccator, which was provided with a beaker of concentrated sulfuric acid and one of sodium hydroxide flakes as desiccants.

AMINO ACID ANALYSES OF PEPTIDE HYDROLYSATES

Dried hydrolysates were dissolved in 0.25 ml of an aqueous solution containing 0.125 μmoles each of cysteic acid and of norleucine. Cysteic acid and norleucine served as internal references for the analyses. The entire sample of hydrolysate was loaded on one of five 3 mm X 130 cm

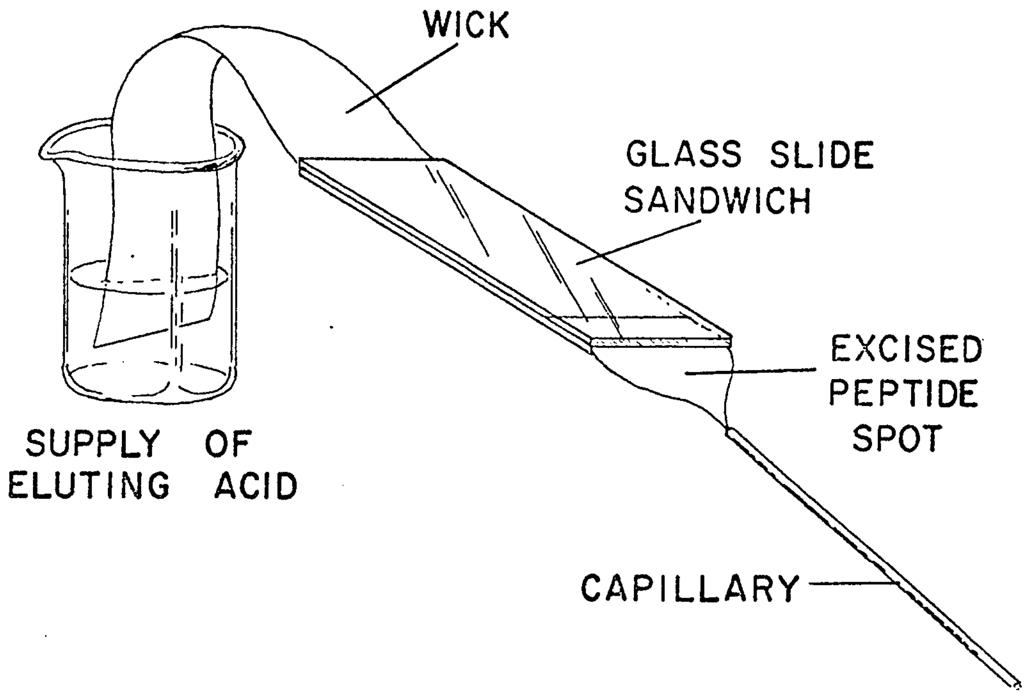

Figure 3. Arrangement for Elution of Peptides from Peptide Maps.

columns of cation exchange resin (Technicon Chromo-Beads, Type A, Lot No. 111A). The Technicon[6] amino acid analyzer allowed up to five columns to be loaded simultaneously and run consecutively. Each analysis required approximately 6½ hours. The column was eluted with non-linear gradient varying in pH and in ionic strength. Each chamber of a nine-chambered varigrad contained 20 ml of eluate. Composition of solutions used in the gradient are shown in Table 1. The nine chambers of the varigrad were filled in the following manner:

Chamber	Volume	Solution	pH
1	20 ml	A	2.75
2	20 ml	B	2.75
3	20 ml	B	2.75
4	15 ml 5 ml	C D	3.8 5.0
5	10 ml 10 ml	C D	3.8 5.0
6	20 ml	C	3.8
7	20 ml	D	5.0
8	20 ml	E	6.1
9	20 ml	E	6.1

A ninhydrin solution was introduced into the lines with the separated amino acids by means of a proportioning pump. Chromatograms were obtained which displayed the various amino acids in terms of the absorbance at 570 nm

[6]Technicon Chromatography Corp., Chauncey, New York.

Table 1. Solutions Used on the Amino Acid Analyzer

Chemicals	Solution A pH 2.75	Solution B pH 2.75	Solution C pH 3.8	Solution D pH 5.0	Solution E pH 6.1
Sodium hydroxide	2.45 g/l	2.45 g/l	2.45 g/l	2.45 g/l	2.45 g/l
Sodium citrate	14.7 g/l	14.7 g/l	14.7 g/l	14.7 g/l	14.7 g/l
BRIJ	10 ml/l	10 ml/l	10 ml/l	10 ml/l	10 ml/l
Thiodi-glycol	5 ml/l	5 ml/l	5 ml/l	----	----
EDTA	0.05 g/l	0.05 g/l	----	----	0.10 g/l
3 N HCl	58 ml/l	56 ml/l	45 ml/l	22.2 ml/l	15 ml/l
T. butyl alcohol	100 ml/l	----	----	----	----
Sodium chloride	----	----	----	35.3 g/l	128.5 g/l

and 440 nm of their products from reaction with ninhydrin as a function of the time of their elution. All of the amino acids except proline are indicated by the absorbance at 570 nm. The product of the reaction of ninhydrin with proline absorbs at 440 nm. The beginning of a chromatogram is marked by the appearance of the internal reference, cysteic acid. It is followed by other amino acids in the following order: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, cysteine, methionine, isoleucine, leucine, reference norleucine, tyrosine, phenylalanine, ammonia, aminoethylcysteine, lysine, histidine, and arginine.

Calculation of Relative Amounts of Amino Acids

The amount of a particular amino acid present in an hydrolysate is indicated quantitatively by the areas of the absorbance peaks on the chromatogram of the hydrolysate. These areas were calculated by multiplying the height of the peak by the "dot width" at half height.

The molar absorbances of the products of the reaction of amino acids with ninhydrin vary according to the amino acid. It therefore was necessary to use "standard" samples on the analyzer to obtain "color values" to calibrate the data. The standard contained 0.1 μmole of each of the 17 common amino acids (excluding tryptophan) and 0.1 μmole each of the internal references norleucine and cysteic acid. Values of peak area from three standards were calculated and averaged every time a new set of reagents were made. This became necessary about every 60 analyses. These areas from the standard were divided by the area of the norleucine peak to give a set of calibration values. When the peak area values from a particular analysis are divided by their respective calibration values from the standards, the result is a set of numbers showing the relative μmolar amounts of the amino acids under consideration. The calibrations numbers from the standards used in this research are shown in Table 2.

After the single amino acids analyses of the hydrolysates of individual peptides were reduced to relative numbers of micromoles, they were empirically fitted to the "best" near integral proportions using a selected divisor.

DETECTION OF TRYPTOPHAN

Tryptophan is completely destroyed during acid hydrolysis of the peptide and therefore must be detected qualitatively through specific staining for tryptophan. Dry peptide maps or, in some cases, wedges from particular spots were dipped in a fresh solution of 1% p-dimethylaminobenzaldehyde (Eastman Kodak) in a mixture of 9 volumes acetone and 1 volume concentrated HCl (Ehrlich's reagent; Smith, 1953). A rose colored spot, which appeared at room temperature within five minutes after the maps were dipped,

Table 2. Calibration Values Obtained from the Analyses of Reference Mixture 0.1 μmolar in Each of the Amino Acids[a]

AA	Identification Numbers of Analyses to Which Calibration Values Apply 2563-2621	2622-2678	2679-2733	2734-2788	2789-2842
B(D,N)	0.81	0.82	0.76	0.80	0.78
T	0.95	0.97	0.85	0.93	0.90
S	0.95	0.98	0.91	0.93	0.94
Z(E,Q)	0.91	0.96	0.84	0.94	0.90
P	0.192	0.206	0.209	0.216	0.210
G	1.24	1.31	1.23	1.22	1.30
A	0.82	0.85	0.79	0.83	0.84
V	0.75	0.79	0.71	0.76	0.76
M	1.05	1.12	1.08	1.08	1.08
I	0.80	0.80	0.75	0.79	0.78
L	1.11	1.08	1.08	1.10	1.09
O	1.12	1.13	1.07	1.11	1.10
F	1.08	1.09	1.01	1.05	1.06
K	1.04	1.03	0.99	1.04	1.06
H	1.24	1.26	1.22	1.25	1.24
R	1.09	1.02	0.98	0.99	1.04
AEC	0.82	0.82	0.82	0.82	0.82

[a]See page 37 for explanation of abbreviations.

indicated the presence of tryptophan. These maps were dried and marked for tryptophan. Several other amino acids which are partially destroyed by acid hydrolysis will be discussed in a later section of this thesis.

RESULTS

Multiple peptide maps of the tryptic digests of the hemeoglobin of _Clethrionomys_ were prepared on each of three occasions during this investigation. The first peptide maps of _Clethrionomys_ Hb-f from which amino acid analyses were taken were made during November, 1968. The method was essentially the same as that described by C. Genaux (1969) with the omission of the derivatization of the globin. The eluted peptides were too low in quantity to give clear results, although several peptides were tentatively identified. Peptide maps were prepared a second time during February, 1969, from the total _Clethrionomys_ Hb. To increase the peak area on the chromatograms from the amino acid analyzer, preparative gel electrophoresis was omitted and therefore globin was precipitated from unresolved components of hemoglobin. This map yielded preliminary identification of about half of the peptides. Although the peptides yielded analyses with greater peak areas, there also appeared to be contamination and/or incomplete separation of the peptides, and the resulting stoichiometry was poor. The final maps were prepared in early June, 1969, following the procedure as described in Materials and Methods of this thesis. The duration of electrophoresis and chromatography was increased to obtain better resolution of peptide spots. The analyses from the third pair of maps were of higher quality than those from the previous peptide maps. Unless it is otherwise noted, the experimental data given in Tables 3 through 30 were taken from the amino acid analyses of hydrolysates from the third pair of maps prepared during June, 1969. Preliminary identifications of many peptides were only substantiated by the data taken from the third maps.

PEPTIDE MAPS OF TRYPTIC DIGESTS OF HEMOGLOBIN OF CLETHRIONOMYS AND TWO OTHER REFERENCE MICROTINES

Fig. 4 shows a tracing of the peptide map of the separated tryptic peptides of the major hemoglobin component of *Clethrionomys*. The spots are numbered according to the tryptic peptides of the alpha and beta chains of hemoglobin which they represent. Some spots, representing unidentified peptides, were labeled with numbers only. Fig. 5 shows the relative positions of the peptides of the alpha chain of the two reference *Microtus* hemoglobins, of *Clethrionomys* Hb-f and of human hemoglobin. Fig. 6 compares the positions for the peptides of the beta chain. These composite maps were prepared by superpositions of the four maps.

A number of abbreviations and symbols are used in the following pages. They are as follows:

Hb	Hemoglobin
MP	*Microtus pennsylvanicus* Hb
MAf	*Microtus abbreviatus* Hb-f (fast component of two)
Cf	*Clethrionomys* Hb-f

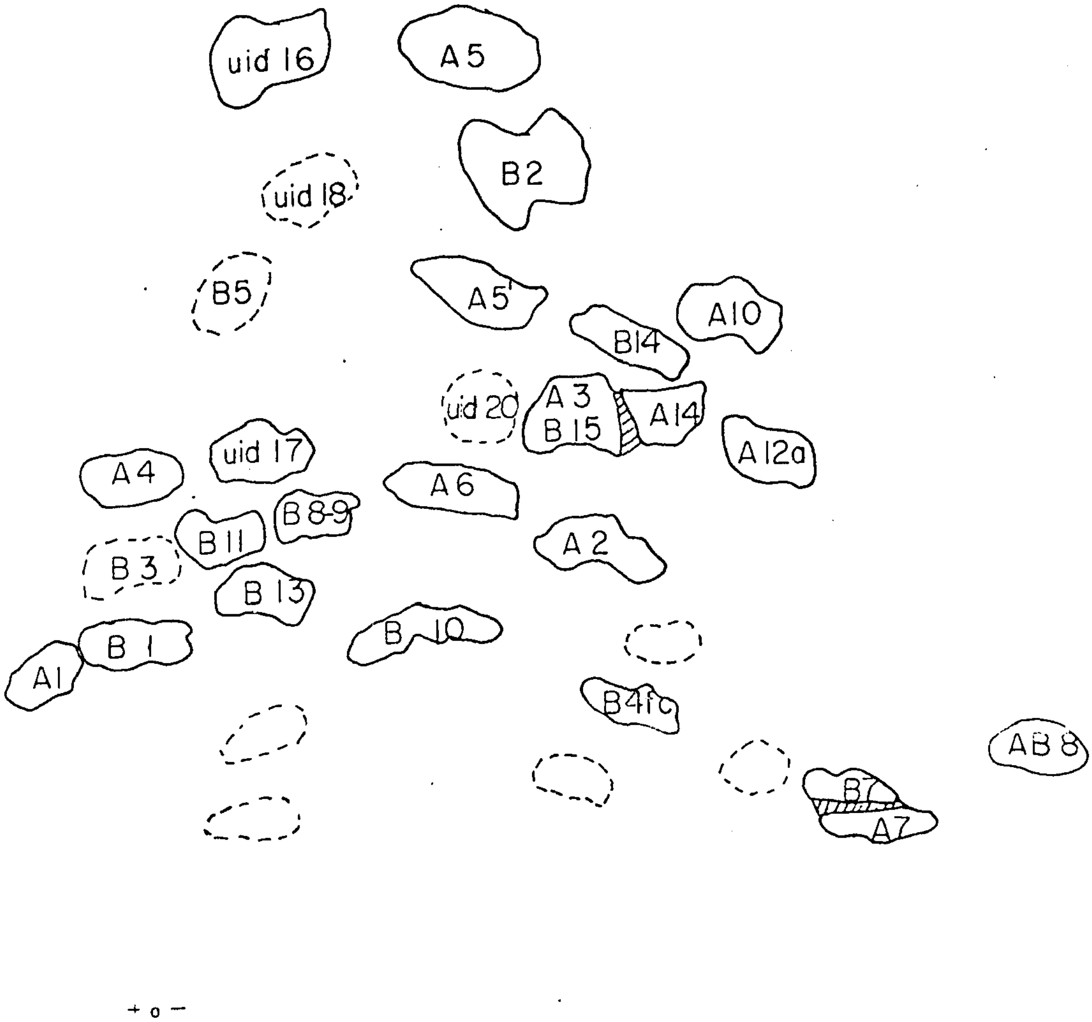

Figure 4. Map of Tryptic Peptides from Hemoglobin-f of <u>Clethrionomys rutilus</u>.

Tracing is reduced approximately X ½
Refer to text for abbreviations.

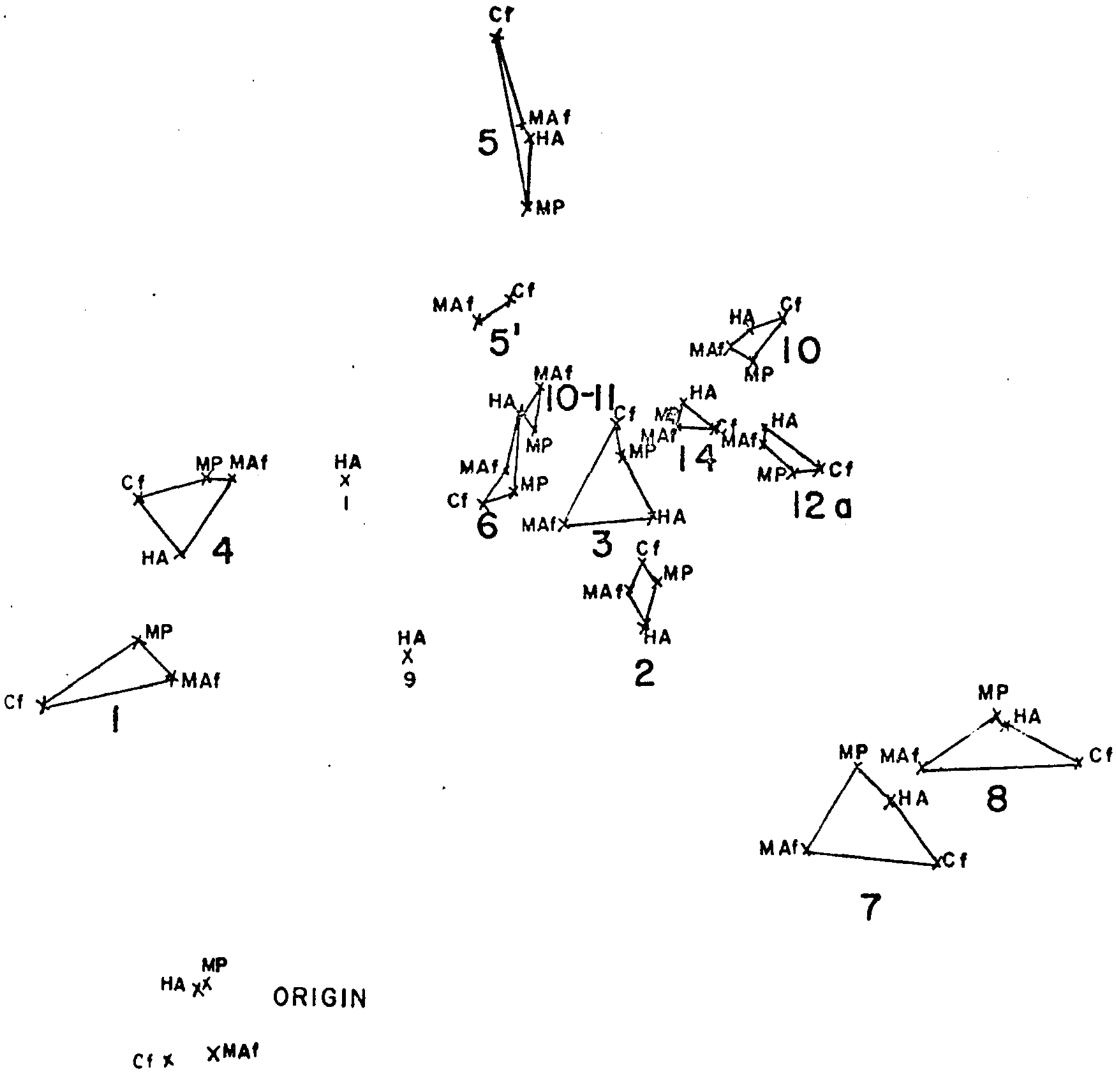

Figure 5. Composite Map of the Tryptic Alpha Peptides from the Hemoglobins of-

Cf Clethrionomys (Hb-f)

MP Microtus pennsylvanicus

MAf Microtus abbreviatus (Hb-f)

HA Human

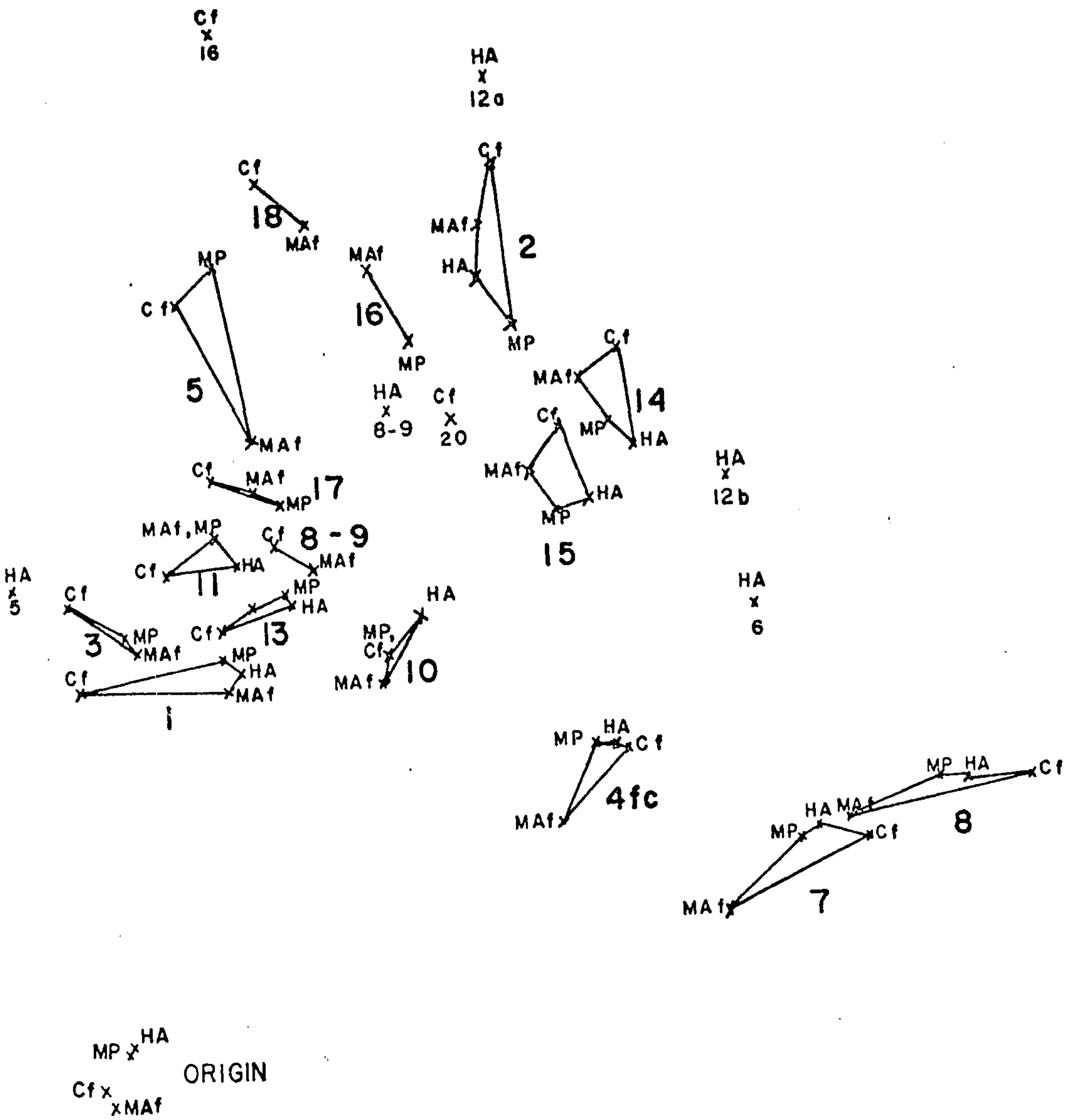

Figure 6. Composite Map of the Tryptic Beta Peptides from the Hemoglobins of-

Cf	Clethrionomys (Hb-f)
MP	Microtus pennsylvanicus
MAf	Microtus abbreviatus (Hb-f)
HA	Human

A1 is the designation of the tryptic peptide alpha-T1 and refers to the first tryptic peptide from the N-terminal of the alpha chain. Likewise A2 refers to the second tryptic peptide of the alpha chain while B2 refers to the second tryptic peptide from the N-terminal of the beta chain.

In some cases only a fragment of a peptide was detected. One of these, B4fc was a fragment of the C-terminal end of beta-T4. Other fragments are designated by the letters a and b and fn. The letters uid refer to an unidentified peptide.

COMPARATIVE AMINO ACID COMPOSITION OF THE PEPTIDES SEPARATED FROM TRYPTIC HYDROLYSATES OF HEMOGLOBIN FROM THREE MICROTINES.

The results of the amino acid analyses of the eluted and hydrolyzed ninhydrin-positive peptides are given in Tables 2 through 30. For comparison purposes, data for the hemoglobins of *M. pennsylvanicus* and *M. abbreviatus* (fast component) are included in the tables. Reference data for the two *Microtus* are taken from the PhD thesis of C. Genaux (1969).

Single letter abbreviations are used in Tables 2 through 30 to designate the amino acids according to Dayhoff and Eck (1968):

K Lysine	R Arginine
H Histidine	D Aspartic Acid

N	Asparagine	A	Alanine
B	Either D or N, not distinguished	V	Valine
T	Threonine	M	Methionine
S	Serine	I	Isoleucine
E	Glutamic Acid	L	Leucine
Q	Glutamine	O	Tyrosine
Z	Either E or Q, not distinguished	F	Phenylalanine
P	Proline	C	(Aminoethyl-) Cysteine
G	Glycine	W	Tryptophan

The word TRACE signifies that qualitative evidence was observed for the presence of the amino acid.

The letters AA stand for amino acid. In the tables the identifying number for the single amino acid analysis is given at the bottom of each column of data to which it pertains together with the total micromoles of peptide recovered in that analysis.

Tryptic Peptides of the Alpha Chain of Hemoglobin

Alpha-T1. See Table 3. The peptide alpha-T1 of *Clethrionomys* Hb-f appears to be comprised of the same seven amino acids as that of alpha-T1 of the two *Microtus* Hemoglobins. Consistent with their composition, the peptides are in the same relative position on all three peptide maps.

Alpha-T2. See Table 4. Peptides alpha-T2 of *Clethrionomys* Hb-f and *M. abbreviatus* Hb-f contain the same four amino acids. They contain one more threonine and one less serine than alpha-T2 of *M. pennsylvanicus*. This apparent

Table 3. Amino Acids (AA) Composition of Tryptic Hemoglobin Peptides αT1 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf
K	0.91	0.68	0.76
H			
R			
B	2.17	2.18	1.59
T			
S	1.06	0.90	1.11
Z			
P			
G	1.09	1.04	1.24
A			
C			
V	1.08	0.66	0.92
M			
I			
L	0.96	0.88	1.03
O			
F			
W			
μmole:	0.020	0.024	0.016
Anal.:	1992	2041	2792

Table 4. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT2 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MA f	Cf
K	0.86	0.89	0.90
H			
R			
B	1.08	1.20	1.12
T		0.69	0.54
S	0.79		
Z			
P			
G			
A			
C			
V			
M			
I	1.02	0.94	1.43
L			
O			
F			
W			
μmole:	0.028	0.025	0.020
Anal.:	2003	2044	2791

replacement of one amino acid does not result in any significant displacement in position on the peptide maps.

Alpha-T3. See Table 5. Peptides alpha-T3 and beta-T15 are coincident with one another. Such coincidence was also observed in the case of alpha-T3 and beta-T15 of *M. pennsylvanicus* Hb. There appears to be some contamination by the adjacent peptides alpha-T14 and beta-T14.

Qualitative evidence for the presence of tryptophan was obtained by specific staining for tryptophan on the peptide map. (See Materials and Methods).

Peptides alpha-T3 from *Clethrionomys* Hb-f and *M. pennsylvanicus* seem to have the same amino acid composition and are in the same position on the peptide maps. Relative to *M. abbreviatus*, alpha-T3 of *Clethrionomys* Hb-f has one more threonine and one less aspartic acid. Peptides alpha-T3 of *Clethrionomys* Hb-f and *M. pennsylvanicus* Hb are displaced in the chromatographic direction from the position of alpha-T3 of *M. abbreviatus* Hb-f.

Alpha-T4. See Table 6. The position of the peptides alpha-T4 are the same for *Clethrionomys* Hb-f and for the two *Microtus* hemoglobins. Among the 15 amino acids of peptide alpha-T4, *Clethrionomys* Hb-f lacks tyrosine as compared to the other reference peptides. There appear to be five residues of alanine in this *Clethrionomys Hb* peptide as compared with four and three respectively in the corresponding peptides of the *M. pennsylvanicus* and *M. abbreviatus*

Table 5. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT3 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf	Cf[b]
K	1.8/2 (1.8)	0.88	0.88
H	(0.33)		[0.97]
R			(0.19)
B	(1.20)	0.84	(0.13)
T	2.3/2		0.48
S			
Z			
P			
G	2.0/2 (1.2)	1.06	1.39
A	2.0/2 (0.3)	1.18	1.08
C			
V	(0.60)		(0.17)
M			
I	(0.50)		
L	(1.1)		
O	(1.0)		[0.36]
F	(0.60)		
W	+	+	+
μmole:	0.020	0.033	0.033
Anal.:	2007	2043	2787

[a]Numbers in parentheses pertain to coincident peptides βT15, 16'.
[b]Numbers in parentheses pertain to the presence of some αT14 and βT14. Numbers in brackets pertain to coincident βT15.

Table 6. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT4 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf[a]	Cf[a]
K			
H	1.08	1.01	0.73
R	1.24	1.04	0.70
B			(0.29)
T			
S			
Z	3.20	2.96	3.21
P			
G	3.20	3.70	3.42
A	3.80	3.48	4.75
C			
V		(0.30)	(0.29)
M			
I	0.74	0.88	0.53
L	1.34	1.10	1.07
Q	0.57	0.64	
F			
W			
μmole:	0.028	0.032	0.021
Anal.:	2030	2034	2794

[a] Numbers in parentheses pertain to amino acids not considered to belong to αT4.

hemoglobins. The additional amount of glycine and its possible origin will be discussed in a later section.

Alpha-T5. See Tables 7 and 8. This peptide from *Clethrionomys* Hb-f is composed of the same nine amino acids as the reference peptides. Map positions are identical for the peptides alpha-T5 from hemoglobins of each of the species. On the peptide map, peptide alpha-T5 of *Clethrionomys* Hb-f stained lightly with ninhydrin, although analyses showed the quantity of material to be comparable to other peptides which stained more heavily.

Other peptides, isolated from the peptide maps of *Clethrionomys* Hb-f and of *M. abbreviatus* Hb-f, have a position consistent with alpha-T5 in the chromatographic direction. These peptides are designated alpha-T5'. The data given in Table 8 for *Clethrionomys* are taken from analyses of the peptide map of February, 1969.

Alpha-T6. See Table 9. The same 16 amino acids appear to comprise the peptide alpha-T6 in hemoglobins from all three animals. The peptides were in the same relative position on the peptide maps of both the *Microtus* Hb and *Clethrionomys* Hb-f. Data given in Table 9 for alpha-T6 of *Clethrionomys* Hb were obtained from the analyses of the peptide map of February, 1969. These data have been included because of an analytical failure in the analysis of the peptide from the map of June, 1969.

Table 7. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT5 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf	Cf[a]
K	0.87	1.09	0.89
H			
R			
B			
T	2.04	1.95	1.89
S	(0.4)		
Z			
P	1.05	0.94	1.16
G			(0.47)
A	1.22	1.24	1.42
C			
V	1.15	1.17	1.12
M	0.16	0.27	0.18
I			
L			
O	0.62	0.79	0.78
F	0.80	1.20	0.98
W			
μmole:	0.018	0.016	0.013
Anal.:	2036	2065	2827

[a] Numbers in parentheses pertain to amino acids not considered to belong to αT5.

Table 8. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptide αT5' (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf[b]	Cf[b]
K		0.84	0.80
H			
R			
B			
T		2.02	1.60
S		(0.5)	
Z			
P		0.74	1.00
G		(0.2)	(0.58)
A		1.20	1.18
C			
V		1.01	1.39
M		TRACE	0.36
I			
L			
O		0.56	0.68
F		0.84	0.98
W			
µmole:		0.010	0.011
Anal.:		2060	2591

[a] Corresponding peptide was not found for M. pennsylvanicus.
[b] Numbers in parentheses pertain to amino acids not belonging to peptide αT5'.

Table 9. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT6 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf
K	1.00	0.86	0.83
H	2.07	2.06	1.82
R			
B	1.21	1.08	1.32
T	0.92	1.08	0.53
S	2.10	2.02	1.96
Z	1.10	1.03	1.00
P	0.92	1.12	1.16
G	1.10	1.14	1.19
A	1.19	1.12	1.14
C			
V	2.02	1.93	1.84
M			
I			
L			
O	0.16	0.62	0.71
F	1.67	1.95	1.96
W			
μmole:	0.016	0.031	0.017
Anal.:	2009	2053	2611

Alpha-T7. See Table 10. Peptide alpha-T7 of *Clethrionomys* Hb-f is separate from and directly below that of beta-T7 on the peptide map. While C. Genaux (1969) obtained a similar separation on the peptide map of human Hb, this was not the case for *Microtus* Hb. Although the relative positions of alpha-T7 on the peptide map are the same, the *Clethrionomys* Hb peptide contains one more glycine and one less alanine than the corresponding *Microtus* peptides alpha-T7.

Alpha-T8 and Beta-T8. See Table 11. Free lysine was found in a spot above and to the right of alpha-T7. Its presence is attributed to the "peptide" alpha-T8 and beta-T8. This "peptide" had been found on the map run in February, 1969, but was lost on the June map because of the longer duration of electrophoresis.

Alpha-T9. Peptide alpha-T9 was not detected in hemoglobin maps from any of the three species.

Alpha-T10. See Table 12. Dipeptide alpha-T10 contains the two amino acids arginine and leucine in all three hemoglobins for which data are given in Table 12. Glycine was rejected as possibly belonging to alpha-T10 of *Clethrionomys* Hb-f for reasons to be discussed later. Positions of the peptides alpha-T10 are similar for the two *Microtus* hemoglobins and for *Clethrionomys* Hb-f.

Alpha-T11. On the basis of available analyses, peptide alpha-T11 was not detected on the peptide map of *Clethrionomys*

Table 10. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT7 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	Maf[b]	Cf
K	4.04/2	2.00/2	1.00
H	2.29/2	1.92/2	1.09
R			
B			
T			
S			
Z			
P			
G	1.90/2	2.12/2	1.82
A	1.55/2	1.54/2	
C			
V			
M			
I			
L			
O			
F			
W			
μmole:	0.010	0.092	0.030
Anal.:	2069	2199	2785

[a]Analysis was obtained from coincident peptides αβT7-8.
[b]Numbers for MAf pertain to coincident peptides αβT7.

Table 11. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αβT8 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf	Cf
K		1.00	1.00
H			
R			
B			
T			
S			
Z			
P			
G			
A			
C			
V			
M			
I			
L			
O			
F			
W			
μmole:		0.015	0.050
Anal.:		2095	2463

[a]No data were available for this peptide.

Table 12. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT10 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K			
H			
R	1.10	1.24	1.02
B			
T			
S			
Z			
P			
G			(0.55)
A			
C			
V			
M			
I			
L	0.81	0.55	0.40
O			
F			
W			
μmole:	0.019	0.017	0.022
Anal.:	1949	1999	2768

[a]Number in parentheses pertains to an amino acid not considered to belong to αT10.

Hb-f. The peptide was identified in combination with peptide alpha-T10 on the peptide maps of M. abbreviatus Hb-f and M. pennsylvanicus Hb, although they were recovered in only small amounts.

Alpha-T12. See Table 13. Only a fragment of the peptide alpha-T12 was identifiable. Peptides which represent the same N-terminal fragments of alpha-T12 were obtained from identical positions on all maps. These peptides appear to be composed of the same five amino acids. The value for leucine for the Clethrionomys Hb-f peptide is much lower than would be expected to represent two residues of this amino acid per peptide. Again glycine as a component of this peptide cannot be ruled out completely; reasons against its inclusion as a component will be presented in the discussion.

Alpha-T13. Complete peptides alpha-T13 were not identified. No evidence was found for this peptide in tryptic digests of Clethrionomys Hb-f or Microtus hemoglobin. Three chymotryptic peptides obtained from the tryptic residues from M. pennsylvanicus hemoglobin were estimated as fragments of alpha-T12 and alpha-T13.

Alpha-T14. See Table 14. Dipeptide alpha-T14 of Clethrionomys Hb-f occupies the same positions on its peptide maps as that occupied by alpha-T14 on the two maps from Microtus hemoglobin. The three hemoglobin peptides

Table 13. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides αT12a (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K			
H	1.03	1.16	1.10
R			
B			
T			
S	1.12	1.12	1.26
Z			
P			
G			(0.55)
A			
C	0.25	TRACE	TRACE
V			
M			
I			
L	1.78	1.76	1.50
O			
F			
W			
μmole:	0.014	0.015	0.019
Anal.:	1990	2176	2762

[a]Number in parentheses pertains to an amino acid not considered to belong to αT12a.

Table 14. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptide αT14 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K			(0.15)
H			(0.17)
R	1.00	0.90	1.14
B			
T			
S			(0.16)
Z			
P			
G			(0.27)
A			(0.35)
C			
V			(0.25)
M			
I			
L			(0.11)
O	0.59	0.43	0.47
F			
W			
μmole:	0.028	0.034	0.028
Anal.:	2001	2051	2781

a Numbers in parentheses pertain to presence of some peptide β14.

appear to have the same two major constituent amino acids, although the *Clethrionomys* Hb-f peptide is considered to be incompletely separated from the adjacent peptide beta-T14.

Tryptic Peptides of the Beta Chain of Hemoglobin

Beta-T1. See Table 15. Peptide beta-T1 of *Clethrionomys* Hb-f appears to have the same eight amino acids and peptide map position as beta-T1 for the two *Microtus* hemoglobins. On the peptide map of *Clethrionomys* Hb-f there apparently was some overlap with alpha-T1 and an additional amount of serine and glycine.

Beta-T2. See Table 16. Peptides beta-T2 have the same nine amino acids and the same relative map positions for the *Clethrionomys* Hb-f and for the two *Microtus* Hb. The presence of tryptophan was determined qualitatively by specific staining of a separate peptide map for tryptophan. The data, representing two residues of alanine, are low for all three hemoglobin peptides.

Beta-T3. See Table 17. The composition of peptide beta-T3 from *Clethrionomys* Hb-f appears to differ from that from the two *Microtus* Hb. Data for *Clethrionomys* Hb-f indicate that beta-T3 contains one more glycine, one more valine and two less alanine than beta-T3 of the hemoglobins from the reference animals. These differences do not seem to alter significantly the position of the peptide on the different maps. The adjacent *Clethrionomys* Hb-f peptides

Table 15. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT1 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K	1.06	1.03	0.70 (0.30)
H	0.83	0.86	0.67
R			
B	1.08	1.20	1.00 (0.62)
T	0.92	1.10	0.94
S			(0.81)
Z	1.12	1.06	1.04
P			
G			(0.90)
A	1.11	1.24	1.22
C			
V	0.73	0.69	0.66 (0.37)
M			
I			
L	0.52	1.14	1.00 (0.39)
O			
F			
W			
μmole:	0.018	0.032	0.023
Anal.:	2005	2061	2793

[a] Numbers in parentheses pertain to the presence of some αT1 and additional S and G.

Table 16. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT2 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf
K	0.82	1.04	0.86
H			
R			
B			
T			
S	0.92	1.08	1.18
Z			
P			
G	1.97	2.12	2.08
A	1.56	1.69	1.52
C			
V			
M			
I	0.96	1.13	0.94
L	1.07	1.13	0.94
O			
F			
W	+	+	+
μmole:	0.018	0.026	0.016
Anal.:	2038	2059	2797

Table 17. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT3 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K			(0.27)
H			(0.27)
R	0.87	1.00	0.92
B	2.11	2.22	1.95 (0.54)
T			
S		(0.10)	
Z	1.12	1.14	1.18 (0.28)
P			(+)
G	1.79	1.96	3.00 (0.28)
A	4.76	4.62	3.10 (0.28)
C			
V	1.03	1.11	1.86 (0.28)
M			
I			
L	1.03	1.28	1.00 (0.28)
O			
F			(0.28)
W			
μmole:	0.015	0.048	0.035
Anal.:	2023	2033	2775

[a] Numbers in parentheses pertain to coincident peptide βT11.

beta-T11 and beta-T3 seem to be incompletely separated from one another.

Beta-T4. See Table 18. It was not possible to locate a complete peptide beta-T4 composed of ten amino acids. However, peptides containing three amino acids were found and were labeled beta-T4fc since their analyses are consistent with a C-terminal fragment from beta-T4. Work with human hemoglobin by C. Genaux (1969) in the same laboratory was valuable for the identification of this peptide. All peptides beta-T4fc in Table 18 appear to be identical both in map location and in composition with the exception of small amounts of glycine and serine which appear in the *Clethrionomys* Hb-f analyses.

Beta-T5. See Table 19. This peptide of *Clethrionomys* Hb-f contains 19 amino acids, but its composition corresponds neither to that of the *M. pennsylvanicus* Hb peptide nor to that of the *M. abbreviatus* Hb-f peptide. Its position on the peptide map is further in the chromatographic direction than peptide beta-T5 of *M. abbreviatus* Hb-f and not as far in the chromatographic direction as beta-T5 of *M. pennsylvanicus* Hb. Peptide beta-T5 of *Clethrionomys* Hb-f appears to have one more glutamic acid and one less serine than the corresponding peptide of *M. pennsylvanicus* Hb. It differs in two amino acids from the corresponding peptide of *M. abbreviatus* Hb-f. The *Clethrionomys* peptide shows one more glutamic acid and one more phenylalanine, one less threonine

Table 18. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT4fc (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K			
H			
R	0.96	1.12	1.01
B			
T	0.76	0.69	0.53
S			(0.23)
Z	1.14	1.14	1.23
P			
G			(0.31)
A			
C			
V			
M			
I			
L			
O			
F			
W			
μmole:	0.022	0.036	0.031
Anal.:	2025	2063	2779

[a] Numbers in parentheses pertain to amino acids not considered to belong to βT4fc.

Table 19. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT5 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf
K	1.03	0.79	0.67
H	1.21	1.22	0.94
R			
B	1.83	2.00	2.04
T		0.52	
S	2.57	1.92	2.30
Z	1.34	1.05	1.86
P		TRACE	
G	2.19	1.68	1.92
A	2.82	3.02	2.76
C			
V	1.82	1.52	1.59
M	0.61	0.28	0.31
I			
L	1.02	1.93	1.14
O			
F	2.10	1.29	1.59
W			
μmole:	0.005	0.005	0.015
Anal.:	1923	2211	2814

and one less leucine than beta-T5 of _M. abbreviatus_ Hb-f.

Beta-T6. Peptide beta-T6 was not detected on hemoglobin peptide maps from any of the three species. It is a prominent dipeptide in the map of human hemoglobin.

Beta-T7. See Table 20. Peptide beta-T7 was completely separate from and directly above peptide alpha-T7 on the peptide map. The peptide appears to be comprised of the same four amino acids as those of the _Microtus_ hemoglobins. The data for alanine are low.

Beta-T8. See alpha-T8.

Beta-T9. See Table 21. No peptide of 16 amino acids could be identified as beta-T9. However, a peptide of 17 amino acids containing two lysine residues was detected in an identical position on maps of both _M. abbreviatus_ Hb-f and _Clethrionomys_ Hb-f. The analyses are consistent with a peptide beta-T(8-9). The work of C. Genaux (1969) with human hemoglobin was useful in this case for the identification of this peptide. Relative to beta-T(8-9) of _M. abbreviatus_ Hb-f, the _Clethrionomys_ Hb-f peptide appears to have one less alanine and one more phenylalanine.

Beta-T10. See Table 22. This 13 amino acid peptide of _Clethrionomys_ Hb-f is in the same relative map position as peptide beta-T10 of _M. abbreviatus_ Hb-f and is slightly slower than beta-T10 of _M. pennsylvanicus_ Hb in the chromatographic direction. Consistent with its position, beta-T10 of _Clethrionomys_ Hb-f has the same composition as the peptide

Table 20. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT7 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MA f[b]	Cf
K	4.04/4	2.00/2	0.99
H	2.29/2	1.92/2	1.01
R			
B			
T			
S			
Z			
P			
G	1.90/2	2.12/2	1.31
A	1.55/2	1.54/2	0.60
C			
V			
M			
I			
L			
O			
F			
W			
μmole:	0.010	0.092	0.030
Anal.:	2069	2199	2780

[a] Analysis was obtained from coincident peptides αβT7-8.
[b] Numbers pertain to coincident peptides αβT7.

Table 21. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT(8-9) (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf[b]	Cf[b]
K		1.81	1.86
H		1.20	1.21
R			
B		4.00	4.00
T		0.63	0.36
S		0.64	0.67
Z			(0.26)
P			
G		0.53	0.72
A		1.72	1.00
C			
V		0.52	0.53
M			
I			
L		4.16	4.06
O			
F		(0.20)	0.35
W			
μmole:		0.027	0.016
Anal.:		2142	2760

a This peptide not identified in terms of available analyses.
b Numbers in parentheses pertain to amino acids not considered to belong to βT(8-9).

Table 22. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT10 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K	0.88	1.11	0.90
H	0.93	1.07	1.00
R			
B	1.20	1.24	1.08
T	0.98	1.04	0.90
S	1.94	2.76	2.72
Z	1.13	1.28	1.15
P			
G	0.79	0.54	0.70
A	1.14		(0.27)
C	0.52	0.44	0.40
V			
M			
I			
L	1.82	1.89	2.00
O			
F	0.85	0.89	1.00
W			
μmole:	0.021	0.026	0.018
Anal.:	1991	2079	2763

a Number in parentheses pertains to an amino acid not considered to belong to βT10.

of *M. abbreviatus* Hb-f, but has one less alanine and one more serine than the peptide of *M. pennsylvanicus* Hb.

Beta-T11. See Table 23. Peptides beta-T11 were isolated from tryptic digests of *Microtus* and *Clethrionomys* hemoglobins in similar map positions. Relative to the two reference peptides, beta-T11 of *Clethrionomys* Hb-f appears to have one more lysine and one less arginine. Conspicuous in all three peptides are additional amounts of glycine and alanine which are not attributed to the beta-T11 peptide.

Beta-T12. This peptide was not located on either of the *Microtus* Hb maps or on the *Clethrionomys* Hb-f maps.

Beta-T13. See Table 24. Peptide beta-T13 of *Clethrionomys* Hb-f contains the same 12 amino acids as that of the corresponding *M. pennsylvanicus* Hb peptide. Relative to the corresponding *M. abbreviatus* Hb-f peptide, it has one more serine and one more leucine, one less alanine and one less phenylalanine. The peptides beta-T13 were all isolated from the same relative position on the peptide maps.

Beta-T14. See Table 25. Hemoglobin peptides beta-T14 from *Clethrionomys* and from the two *Microtus* contain the same 12 amino acids and are found in the same relative map positions. Values for valine which represent three amino acids per peptide are low for all three animals.

Table 23. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT11 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf[a]	Cf[a]
K			0.75
H	1.11	0.96	0.93
R	0.86	0.76	(0.21)
B	2.21	1.88	2.25
T			(0.36)
S		(0.40)	(0.37)
Z	1.16	1.01	1.02
P	1.06	0.88	0.78
G	(0.30)	(0.70)	(0.98)
A	(0.70)	(0.90)	(1.33)
C			
V	1.03	0.92	1.17
M			
I			
L	0.86	0.86	1.16
O			
F	0.82	0.82	0.77
W			
μmole:	0.031	0.015	0.034
Anal.:	1969	2029	2757

a Numbers in parentheses pertain to presence of some αT4.

Table 24. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT13 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf[a]	Cf[a]
K	1.20	1.09	0.99
H			(0.47)
R			
B	1.14	0.91	1.00
T	1.22	1.17	1.04
S	0.60	(0.31)	1.06
Z	1.63	2.19	2.02
P	0.62	1.14	0.80
G	(0.38)	(0.45)	(0.45)
A	3.06	4.05	2.66
C			
V	(0.31)		(0.41)
M			
I			
L	0.58	(0.45)	0.86
O			
F	1.30	1.82	1.52
W			
μmole:	0.018	0.018	0.037
Anal.:	2031	2200	2752

[a] Numbers in parentheses pertain to amino acids not considered to belong to βT13.

Table 25. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT14 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf
K	1.12	1.04	0.90
H	1.28	1.14	1.00
R			
B			
T			
S	1.09	1.07	1.17
Z			
P			
G	1.13	1.08	1.28
A	4.28	4.40	4.16
C			
V	2.48	2.28	2.21
M			
I			
L	1.12	1.15	1.10
O			
F			
W			
μmole:	0.017	0.026	0.017
Anal.:	2000	2184	2788

Beta-T15. See Table 26. Dipeptides beta-T15 of identical composition were found in the same map position for the hemoglobins from both reference animals and from _Clethrionomys_. Like _M. pennsylvanicus_ Hb, peptide beta-T15 of _Clethrionomys_ Hb-f was found coincident with peptide alpha-T3. Peptides beta-T15 from the _Microtus_ hemoglobins were also found coincident with an unidentified peptide designated 16'.

Unidentified Peptides

A number of peptide spots taken from maps of tryptic digests of _Clethrionomys_ Hb-f yielded amounts of amino acids which were insufficient for identification purposes. In addition to these analyses several adequate analyses were obtained from the _Microtus_ hemoglobins and from the _Clethrionomys_ Hb-f, but it has not been possible to correlate the analyses with specific segments of the hemoglobin alpha or beta chains.

Peptide 16. See Table 27. Unidentified peptides, designated 16, were obtained from both _Microtus_ Hb and _Clethrionomys_ Hb-f peptide maps. Peptides 16 of _M. pennsylvanicus_ Hb and _M. abbreviatus_ Hb-f appear to be composed of the same ten amino acids and are found in the same relative map position. Peptide 16 of _Clethrionomys_ Hb-f is found further in the chromatographic direction and not as far in the electrophoretic direction as peptide 16 of the two _Microtus_ hemoglobins. Relative to the _Microtus_ peptides 16,

Table 26. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptides βT15 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf[b]	Cf[c]
K			
H	1.00	1.00	1.00
R			
B			
T			
S			
Z			
P			
G			
A			
C			
V			
M			
I			
L			
O	0.70	0.60	0.37
F			
W			
μmole:	0.020	0.018	0.034
Anal.:	2007	2049	2787

a Numbers taken from data for coincident peptides 16', αT3, and βT15.
b Numbers taken from data for coincident peptides 16' and βT15.
c Numbers taken from data for coincident peptides αT3 and βT15.

Table 27. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptide 16 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf
K	0.95	0.98	0.75
H	0.99	1.06	
R			
B	1.13	1.18	1.10
T			0.91
S			
Z			
P			
G	0.98	1.02	1.83
A	1.92	2.23	1.05
C			
V	0.45	0.46	0.45
M			
I	0.50	0.63	0.63
L	1.11	0.74	1.07
O			
F	1.00	1.08	0.86
W			
μmole:	0.012	0.016	0.028
Anal.:	2040	2067	2796

the unidentified peptide of _Clethrionomys_ Hb-f appears to have one less histidine and one less alanine, one more threonine and one more glycine per peptide.

Peptide 17. See Table 28. Other unidentified peptides, designated 17, are located near beta-T11 in the same region of all three peptide maps. If the possibility of other overlapping peptides is neglected, peptide 17 of _Clethrionomys_ Hb-f and _M. abbreviatus_ Hb-f could be composed of the same 17 amino acids. Also neglecting the possibility of overlapping peptides, peptide 17 of _M. pennsylvanicus_ Hb appears to be composed of 22 amino acids which include the five additional amino acids: aspartic acid, proline, valine, lysine and possibly phenylalanine.

It should be noted that the micromolar amount of peptide 17 of _Clethrionomys_ Hb-f is almost twice that of other peptides of _Clethrionomys_ Hb-f. This may be evidence for the existence of more than one peptide at this location.

Peptide 18. See Table 29. Unidentified peptides, designated 18, were obtained from similar positions on the _Clethrionomys_ Hb-f and _M. abbreviatus_ Hb-f peptide maps. Peptide 18 of _Clethrionomys_ Hb-f appears to be composed of 15 amino acids. Peptide 18 of _M. abbreviatus_ appears to be composed of the same 15 amino acids although the stoichiometry does not correspond precisely.

Peptide 20. See Table 30. An unidentified peptide was obtained from _Clethrionomys_ Hb-f in the region of the

Table 28. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptide 17 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP	MAf	Cf[a]
K	1.91	1.04	1.20
H	1.04	1.01	0.83
R	(0.41)	(0.21)	
B	4.07	2.80	2.53
T	0.78	0.54	0.51
S	0.89	0.99	1.00
Z	0.60	0.56	0.55
P	2.09	1.00	1.00
G	0.62	0.86	0.96
A	1.81	1.89	1.84
C			
V	3.20	2.04	2.10
M		+	0.10
I			
L	1.71	1.69	1.73
O			
F	1.51	1.15	1.20
W			
μmole:	0.012	0.028	0.048
Anal.:	2010	2218	2767

[a] The datum for histidine of this peptide is estimated due to a mechanical failure of the AA analyzer.

Table 29. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptide 18 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf	Cf
K		0.84	0.63
H		1.17	0.87
R			
B		1.98	1.97
T		1.05	0.71
S		0.87	0.81
Z			
P		1.63	0.89
G		0.42	0.45
A		2.54	2.04
C			
V		1.20	1.00
M			
I		0.52	0.46
L		1.71	1.97
O			
F		0.55	0.59
W			
μmole:		0.010	0.014
Anal.:		2202	2826

[a] Data were not obtained from the analysis of this peptide.

Table 30. Amino Acid (AA) Composition of Tryptic Hemoglobin Peptide 20 (in Residues of AA per Peptide).

	REFERENCE		EXPERIMENTAL
AA	MP[a]	MAf[a]	Cf
K			1.00
H			1.11
R			(0.33)
B			1.92
T			1.03
S			2.56
Z			1.28
P			+
G			1.22
A			1.00
C			
V			1.86
M			
I			
L			1.30
O			
F			1.92
W			
μmole:			0.008
Anal.:			2798

[a] Data were not obtained from the analysis of these peptides.

map common to peptides alpha-T(10-11) of the *Microtus* hemoglobins. However, the composition of peptide 20 could not be correlated with that of alpha-T(10-11) of the *Microtus* hemoglobins. If the possibility of other overlapping peptides is neglected, peptide 20 could be composed of 15 to 17 amino acids.

DISCUSSION

STOICHIOMETRY OF THE PEPTIDE DATA

Most of the amino acid data in Tables 3 through 30 approximates integral numbers of amino acids per peptide, in accord with the Law of Multiple Proportions, if a latitude of plus or minus 0.3 amino acids is allowed. There are two general explanations which can account for most of the observed departures from perfect stoichiometry: 1) loss of amino acids during analytical procedures, and 2) apparent gain of amino acids in a peptide analysis due to minor or major contaminants which can often be accounted for in terms of a second unresolved, coincident peptide.

Amino Acids with Low Recovery after Hydrolysis

Some amino acids are partially degraded by acid hydrolysis, and the values for these amino acids presented in the data are low. Although generalizations can be drawn about the degree of degradation of a specific amino acid, evidence of a fair recovery of a certain amino acid in one peptide is not inconsistent with a low recovery in another. It has been suggested by G. Tristram (1963) that losses of certain amino acids may be a function of the amino acid composition and sequence.

Cysteine. Cysteine may be destroyed completely during acid hydrolysis. In the attempt to achieve improved recovery of peptides in this research, cysteine was derivatized to aminoethylcysteine. Aminoethylcysteine was detected on the chromatograms of peptides alpha-T12a and beta-T10 in amounts of TRACE and

0.40 residue respectively. Single residues of cysteine are considered to be present in these peptides.

Tyrosine. The amino acid tyrosine possesses a phenolic side chain and is subject to partial destruction during acid hydrolysis. The analyses of peptides alpha-T4, alpha-T5, alpha-T6, alpha-T14 and beta-T15 showed from 0.36 to 0.87 tyrosine residue per peptide. Single residues of tyrosine are considered to be present in each peptide.

Methionine. Methionine is largely destroyed during acid hydrolysis. Peptides containing methionine are alpha-T5, alpha-T5', beta-T5 and unidentified peptide 17. In these peptides numbers for methionine range from TRACE to 0.31. There is considered to be one residue of methionine in each of these peptides.

Methionine may be partly oxidized at its sulfur atom to the sulfone and/or sulfoxide. If that is the case, one might expect to find a methionine-containing peptide as two components with different chromatographic mobilities depending upon the oxidation state of the methionine. Two methionine-containing peptides with the same amino acid composition identifying them both as alpha-T5 were found at two different locations on the peptide map of Clethrionomys Hb-f. The two locations differed only in the chromatographic direction. Genaux (1969) had also observed this on the peptide map of *M. abbreviatus* Hb-f. The slower peptides are designated alpha-T5'. Since peptide beta-T5 also is considered to contain one residue of methionine, one might expect to find a slower component beta-T5'. Indeed C. Baglioni (1961) reported peptide beta-T5 and peptide "beta-T5 oxidized" on the peptide maps of human hemoglobin A. While no second peptide spot yielded analyses identical to those of beta-T5, the unidentified peptide 17 of both Clethrionomys Hb-f and M. abbreviatus Hb-f contains methionine and all the amino acids of beta-T5 as well as additional amino acids. It is possible that the unidentified peptide is a combination of beta-T5' and another peptide whose identity has not yet been deciphered.

Threonine and Serine. Decomposition of serine and threonine during acid hydrolysis has been reported in the literature (Tristram, 1963). Although systematic destruction of serine was not observed in this research, some degradation of threonine may have been observed. In some analyses, fractional values of 0.36 to 0.56 are considered to represent one residue of threonine per peptide; and in the case of beta-T5' a value of 1.60 amino acids was considered to represent two threonines per peptide.

Reaction of Peptides with Ninhydrin

Often peptide data show at least one amino acid with a low non-integral value. Genaux (1960) observed that data from human hemoglobin frequently contained low non-integral numbers of those amino acids which are known to occupy N-terminal (amino-terminal) positions in their respective peptides. This, he hypothesized, was due to degradation of the N-terminal amino acid upon reaction with ninhydrin when the peptide maps were stained for location of the peptide.

When a free amino acid reacts with ninhydrin, oxidative deamination occurs, and the amino acid is thereby destroyed. The resulting ninhydrin product is blue-colored (Ruhemann's purple) and gives excellent quantitative indication of the amount of amino acids present. It is this property of ninhydrin in reaction with primary amines which makes it useful in the amino acid analyzer.

Because ninhydrin produces the typical blue color when reacted with peptides, it is used to locate the position of a peptide on a peptide map. Although the mechanism of the reaction of ninhydrin with a free amino acid under specific conditions has been determined (McCaldin D.J., 1960), there appears to have been little study of the nature of the reaction between ninhydrin and peptides. Since the formation of Ruhemann's purple with free amino acids involves deamination, it is likely that ninhydrin, in its reaction with a peptide, produces some degradation of the N-terminal amino acid of the peptide.

The hypothesis of a degradation of the N-terminal amino acid by ninhydrin has been applied in postulating the proposed sequences of the hemoglobin of Clethrionomys which are to be presented.

Glycine as a Contaminant

Excessive amounts of the amino acid glycine are conspicuous in the data of nine different peptides of *Clethrionomys* Hb-f. The nine peptides are αT5, αT5', αT10, αT12a, βT1, βT3, βT4 fc, βT11, and βT13. Data from the hemoglobins of the two reference *Microtus* contain three peptides: αT5', βT11, and βT13, also with excessive amounts of glycine.

It is perhaps significant that four of the nine peptides with excessive glycine are in the same area directly above the origin. This area may be contaminated with free glycine. One possible source of glycine contamination is the glycine-containing upper buffer in the preparative gel electrophoresis. Glycine from the upper buffer, having migrated through the gel with the hemoglobin, could be present on the peptide map as free glycine or bound to certain peptides.

A second possible source of glycine contamination is that from human fingerprints. Glycine (0.071 μmole per wet thumbprint) and serine (0.106 μmoles per wet thumbprint) are characteristic of hand contamination (P. Hamilton, 1965). Excessive serine appears with excessive glycine in analyses of the three peptides βT1, βT4fc, and βT11.

In the cysteine-containing peptide, alpha-T12a, the glycine was considered excluded as a component of the peptide as it may be a degradation product of the aminoethylcysteine. To test this possibility, 0.10 μmoles of aminoethylcysteine was subjected to the standard hydrolysis at

112°C. for 28 hours. Analysis of the hydrolysate showed both aminoethylcysteine (peak area 5.86) and glycine (peak area 0.62). It is possible that the degradation product was not glycine but had similar elution properties as glycine.

PROPOSED AMINO ACID SEQUENCE OF CLETHRIONOMYS HEMOGLOBIN PEPTIDES ON BASIS OF HOMOLOGY WITH HUMAN HEMOGLOBIN

In the following section of this Discussion, amino acid sequences for peptides of *Clethrionomys* Hb-f are inferred on the basis of their homology with the well-documented sequence of human hemoglobin A (Dayhoff and Eck, 1968). Because of the similarity between the various mammalian hemoglobins, often attributed to a common origin, two peptides with the same amino acid composition are inferred to have the same sequence; furthermore, in a particular peptide differences of amino acid composition between two species are inferred to involve replacements in the sequence of one amino acid by a second amino acid similar to it. The sequences inferred for the hemoglobin of *Microtus pennsylvanicus* by C. Genaux (1969) are also included.

The letter designations explained on page 37 are used to represent the various amino acids. Aspartic acid is designated with a free beta carboxyl group (D), or its amide, asparagine(N) and glutamic acid with a free gamma carboxyl group (E), or its amide, glutamine (Q) on the basis of the relative electrophoretic mobilities observed for the respective human Hb and *Clethrionomys* Hb-f peptides. (See Figures 5 and 6). The letters B and Z are used to represent aspartic acid and glutamic acid, respectively, when there is no electrophoretic evidence about the nature of the side-chain carboxyls. Dots between the letters indicate an inferred sequence. Differences in the sequences between the various hemoglobins are indicated by underlined letters. Normal human hemoglobin A is represented by the letters HA.

Tryptic Alpha Peptides of Clethrionomys Hb-f, M. Pennsylvanicus Hb and Human Hemoglobin

Alpha-T1. The following sequences record two differences in amino acid composition between the human hemoglobin peptide alpha-T1 and the peptides alpha-T1 of *M. pennsylvanicus* Hb and of *Clethrionomys* Hb-f.

	αT1
Cf	V.L.S.<u>G</u>.(<u>D</u>).(D).K
MP	V.L.S.<u>G</u>.(<u>D</u>).(D).K
HA	V L S <u>P</u> <u>A</u> D K

That the peptides alpha-T1 of the hemoglobins of the three Microtines have the same electrophoretic mobility supplies supporting evidence for the presence of two residues of aspartic acid with free carboxyl side chains in these peptides. The two aspartic acid residues of the *Clethrionomys*

Hb-f peptide are represented by 1.59 in the data. Although an intact peptide alpha-T(1-2) was found in the peptide map from human hemoglobin and from _M. abbreviatus_ Hb-f, such a peptide was not detected on the _Clethrionomys_ Hb-f map.

Alpha-T2. The sequences of alpha-T2 record the one difference in amino acid composition between the three hemoglobins.

	αT2
Cf	T.(N).I.K
MP	S.(N).I.K
HA	T N V K

In the data of alpha-T2 of _Clethrionomys_ Hb-f the low value for threonine (0.54) may be interpreted as due to destruction upon acid hydrolysis or to its destruction in reaction with ninhydrin as a consequence of the probable N-terminal position of threonine.

Alpha-T3. The inferred sequence of amino acids for the peptides alpha-T3 of _Clethrionomys_ Hb-f and of _M. pennsylvanicus_ differs at one position from the peptide of human hemoglobin.

	αT3
Cf	T.A.W.G.K
MP	T.A.W.G.K
HA	A A W G K

That threonine is the only amino acid in the data of this peptide of _Clethrionomys_ Hb-f represented by a low value (0.48) again suggests its possible N-terminal position. Threonine has been reported in the N-terminal position of peptide alpha-T3 from rabbit hemoglobin (G. von Ehrenstein, 1966).

Alpha-T4. The composition of *Clethrionomys* Hb-f peptide alpha-T4 is expressed in the following inferred sequence relative to the corresponding peptides of *M. pennsylvanicus* Hb and of human hemoglobin.

	αT4
Cf	I.G.A.H.A.G.(E).A.G.A.(E).A.L.(E).R
MP	I.G.A.H.A.G.(E).O.G.A.(E).A.L.(E).R
HA	V G A H A G E O G A E A L E R

The low value (0.53) for isoleucine in the *Clethrionomys* Hb-f peptide data may reflect the N-terminal position of isoleucine. Isoleucine has been shown to be N-terminal in peptide alpha-T4 of the hemoglobin of white mouse strain C57BL (Popp, 1965). A substitution of alanine for tyrosine in the eighth position of alpha-T4 has been observed in pig hemoglobin (Yamaguchi et al., 1965; Braunitzer and Kohler, 1966).

Alpha-T5. The inferred sequence of amino acids for the peptide alpha-T5 of *Clethrionomys* Hb-f and of *M. pennsylvanicus* differs at three positions from the peptide alpha-T5 of human hemoglobin.

	αT5
Cf	M.F.V.A.O.P.T.T.K
MP	M.F.V.A.O.P.T.T.K
HA	M F L S F P T T K

Positions three and four of the peptide alpha-T5 of <u>Clethrionomys</u> Hb-f may well be A.V. (alanyl-valyl). Alanine has been reported in the third position of peptide alpha-T5 of the hemoglobin of white mouse strain C57BL (Popp, 1965).

<u>Alpha-T6</u>. The following sequences record the one difference in amino acid composition between the human hemoglobin peptide alpha-T6 and the peptides alpha-T6 of <u>M. pennsylvanicus</u> Hb and of <u>Clethrionomys</u> Hb-f.

	αT6
<u>Cf</u>	T.O.F.P.H.F.(D).<u>V</u>.S.H.G.S.A.(Q).V.K
<u>MP</u>	T.O.F.P.H.F.(D).<u>V</u>.S.H.G.S.A.(Q).V.K
<u>HA</u>	T O F P H F D <u>L</u> S H G S A Q V K

The electrophoretic mobilities of all three peptides are identical. Threonine, which is proposed to be the N-terminal amino acid of this peptide is again represented in the data of <u>Clethrionomys</u> Hb-f with a low value (0.53).

<u>Alpha-T7</u>. The following sequences record one difference in amino acid composition between the <u>M. pennsylvanicus</u> Hb peptide alpha-T7 and the peptides alpha-T7 of human hemoglobin and of <u>Clethrionomys</u> Hb-f.

	αT7
<u>Cf</u>	<u>G</u>.H.G.K
<u>MP</u>	<u>A</u>.H.G.K
<u>HA</u>	<u>G</u> H G K

<u>Alpha-T8</u>. "Peptides" alpha-T8 of all three hemoglobins are inferred to consist of a single lysine residue.

	$\alpha T8$
Cf	K
MP	K
HA	K

Alpha-T9. Although the peptide alpha-T9 containing 29 amino acids was detectable by methods used in this research in tryptic digests of human hemoglobin, it could not be identified from the analyses from the peptide maps of the *Microtus* hemoglobins or of *Clethrionomys* Hb-f.

Alpha-T10. There are no differences in composition between the peptides alpha-T10 from *Clethrionomys* Hb-f, human Hb or *M. pennsylvanicus* Hb.

	$\alpha T10$
Cf	L.R
MP	L.R
HA	L R

Alpha-T11. Tryptic hemoglobin peptides alpha-T11 or alpha-T(10-11) have not been identified for *Clethrionomys* Hb-f. The unidentified peptide 20 occupies a map position similar to that occupied by alpha-T(10-11) in the maps of the *Microtus* hemoglobins and of the human hemoglobins.

Alpha-T12a. An N-terminal fragment of peptide alpha-T12, designated alpha-T12a, was detected on the peptide map of the tryptic digest of *Clethrionomys* Hb-f. The inferred sequences for alpha-T12a of both *Clethrionomys* Hb-f and

M. pennsylvanicus Hb reflect the same amino acid composition as that of human hemoglobin.

	αT12a
Cf	L.L.S.H.C
MP	L.L.S.H.C
HA	L L S H C

Alpha-T12b and Alpha-T13. These peptides of human hemoglobin have been located by Carrell and Irvine (1968) from analyses of peptides produced by chymotrypsin acting upon the insoluble material remaining after initial trypsin digestion of the globin. Chymotrypsin digestion was performed in this research using the methods of Carrel and Irvine (1968); however, the analyses were of a quantity insufficient for calculations.

Alpha-T14. Dipeptides alpha-T14 of the several hemoglobins have the same amino acid composition and the sequences are inferred to be the same.

	αT14
Cf	O.R
MP	O.R
HA	O R

Tryptic Beta Peptides of Clethrionomys Hb-f, M. Pennsylvanicus Hb and Human Hemoglobin

Beta-T1. Differences observed in amino acid composition of peptides beta-T1 are summarized with a sequence of amino acids inferred for *Clethrionomys* Hb-f relative to the other hemoglobin peptides beta-T1.

	βT1
Cf	V.H.L.T.A.(E).(D).K
MP	V.H.L.T.A.(E).(D).K
HA	V H L T P E E K

Both acidic amino acids are considered to have the free carboxyl side chains since the mobility of the peptide beta-T1 of *Clethrionomys* Hb-f is identical to the mobility of the same peptide of human hemoglobin.

The sequence of positions five, six and seven of beta-T1 of *Clethrionomys* Hb-f may be (D).(E).A. (aspartyl glutamyl alanyl). Bonaventura and Riggs (1967) reported the sequence of these positions of white mouse strain BALB/cJ to be B Z A in which the nature of the side chains are not specified for aspartic acid (B) and glutamic acid (Z).

Beta-T2. The two differences in amino acid composition are indicated as four differences in the inferred sequences of beta-T2 of *Clethrionomys* Hb-f and of *M. pennsylvanicus* Hb relative to the sequence of beta-T2 of human hemoglobin. The inferred sequence for the *M. pennsylvanicus* peptide indicated below was proposed by C. Genaux (1969) on the basis of homology with the known sequence of the first five amino acids of peptide beta-T2 of the hemoglobin of white mouse strain BALB/cj (Bonaventura and Riggs, 1967). The postulate that alanine is in the N-terminal position is strengthened by the low value, 1.52, representing two alanines per peptide, which could have resulted from destruction of alanine in reaction with ninhydrin.

	βT2
Cf	A.A.I.S.G.L.W.G.K
MP	A.A.I.S.G.L.W.G.K
HA	S A V T A L W G K

Beta-T3. It was not possible to compare the mobilities of peptide beta-T3 of *Clethrionomys* Hb-f and of human hemoglobin since the peptide beta-T3 was not detected on the peptide map of human hemoglobin. Therefore the letters B and Z are used to represent aspartic acid and glutamic acid respectively in the possible sequence inferred for *Clethrionomys* Hb-f and *M. pennsylvanicus* peptides beta-T3. However, since the peptides beta-T3 of the Microtines did not move in the electrophoretic direction, the probable net charge of the peptides is zero. This implies that two of the three acidic amino acids have free carboxyl side chains.

	βT3
Cf	V.B.V.B.A.A.G.G.Z.A.L.G.R
MP	A.B.V.B.A.A.G.A.Z.A.L.G.R
HA	V N V D E V G G E A L G R

Beta-T4. Due to apparent chymotryptic activity of the trypsin used, peptides beta-T4 were cleaved at the carboxyl bond of tryptophan. The N-terminal fragments beta-T4fn may have migrated off the peptide map in the chromatographic direction, for no analyses were found which could be identified as beta-T4fn. Evidence for the presence of the peptide fragment beta-T4fn was obtained from an early map of Clethrionomys Hb-f which had a chromatography of short duration. When the map was stained with Ehrlich's reagent, a spot which stained positive for the presence of tryptophan was detected at the very top of the map. Recent peptide maps of Lemmus Hb and of M. pennsylvanicus Hb digests prepared with a new stock of trypsin contain an intact peptide beta-T4 of identical composition to beta-T4 of human hemoglobin (Genaux; 1970). It is probable that the entire composition of Clethrionomys Hb-f and M. pennsylvanicus peptides beta-T4 are the same as the peptide of Human Hb in view of the above and in view of the similarity in amino acid composition of all beta-T4 peptides that have been tabulated for hemoglobin (Dayhoff and Eck, 1968).

	βT4
Cf	T.(Q).R
MP	T.(Q).R
HA	L L V V O P W T Q R

Beta-T5 and Beta T6. To account for the missing peptide beta-T6 and the presence of an additional histidine in the peptide beta-T5 of M. pennsylvanicus, C. Genaux suggested that the lysine of beta-T5 of human hemoglobin had been replaced by histidine (H) in the peptide of M. pennsylvanicus and that two amino acids of beta-T(5-6) had been deleted. His proposed sequence was supported by the absence of one aspartic acid from the *Microtus* peptide relative to both the human and white mouse hemoglobins. The dipeptide beta-T6 was easily recognized on the peptide map of human hemoglobin but was absent from the peptide maps of *Microtus* and *Clethrionomys* hemoglobins. It is possible that the sequence of beta-T5 of *Clethrionomys* Hb-f resembles that proposed for the peptide of *M. pennsylvanicus*. However, there appears to be an essential difference in amino acid composition between the peptide of *M. pennsylvanicus* Hb and of *Clethrionomys* Hb-f; peptide beta-T5 of *Clethrionomys* Hb-f has one more glutamic acid residue than the peptide of *M. pennsylvanicus*. Therefore an alternate sequence is proposed for beta-T5 of *Clethrionomys* Hb-f. In the data for beta-T5 of *Clethrionomys* Hb-f both valine (V) and phenylalanine (F) are represented by low numbers and either amino acid might be proposed as N-terminal.

```
                          βT5                      |βT6
Cf    V.F.Z.S.F.G.B.L.S.H.A.Z.A.V.M.G.B.A.K
MP    V.F.Z.S.F.G.B.L.S.S.A._.A._.M.G.B.A.H V.K
HA    F.F E S F G D L S T P D A V M G N P K V K
```

Beta-T7. The proposed sequence of beta-T7 of *Clethrionomys* Hb-f is given below with the inferred sequence of beta-T7 of *M. pennsylvanicus* and the known sequence of the peptide of human hemoglobin.

	βT7
Cf	A.H.G.K
MP	A.H.G.K
HA	A H G K

The histidine has not been found to vary from its position in all peptides alpha-T7 and beta-T7 of hemoglobins reported to date; it is the so-called distal histidine at the sixth coordination position of the iron of hemoglobin.

Beta-T(8-9). No data were available for peptide beta-T9 or beta-T(8-9) of *M. pennsylvanicus* Hb, and the inferred sequence of peptide beta-T(8-9) of *M. abbreviatus* Hb-f is used in its place in the sequences of beta-T(8-9) presented below.

	βT8	βT9
Cf	K.V.L.B.T.F.S.B.G.	L.A.H.L.B.B.L.K
MAf	K.V.L.B.T.L.S.B.G.	A.A.H.L.B.B.L.K
HA	K V L G A F S D G	L A H L D N L K

The aspartic acid (B) has been reported in the third position of beta-T9 in many cases tabulated for hemoglobins by Dayhoff and Eck (1968).

Beta-T10. This peptide of *Clethrionomys* Hb-f differs from the peptides beta-T10 of both *M. pennsylvanicus* Hb and human hemoglobin. Serine has bee reported in the fifth position of beta-T10 of bovine fetal hemoglobin (Dayhoff and Eck, 1968).

	βT10
Cf	G.T.F.S.S.L.S.(E).L.H.C.(D).K
MP	G.T.F.A.S.L.S.(E).L.H.C.(D).K
HA	G T F A T L S E L H C D K

Beta-T11. Although there is a small amount of arginine (0.21) in the data of peptide beta-T11 of Clethrionomys Hb-f, the larger value for lysine (0.75) suggests that the C-terminal amino acid of peptide beta-T11 of Clethrionomys Hb-f is lysine. The replacement of the arginine at the C-terminal of peptide beta-T11 of human hemoglobin by lysine has been reported in several hemoglobins tabulated by Dayhoff and Eck (1968) and is suggested from recent data from tryptic digests of Lemmus hemoglobin (Genaux,1970).

	βT11
Cf	L.H.V.(D).P.(E).(N).F.K
MP	L.H.V.(D).P.(E).(N).F.R
HA	L H V D P E N F R

Beta-T12. While the cysteine containing peptide beta-T12 of human hemoglobin was located in two fragments on the peptide map, peptides beta-T12 or fragments were not identified on the peptide maps of the hemoglobins of Microtus or of Clethrionomys. This could be due either to the failure of trypsin to cleave the peptide beta-T12 at aminoethylcysteine or the absence of a cysteine from beta-T12. Hemoglobin peptides beta-T12 of white mouse strains C57BL/6 and SEC have been reported to contain no cysteine (Rifkin et al., 1966).

Beta-T13. A sequence for Clethrionomys Hb-f peptide beta-T13 is proposed below. The L/F designation in the inferred sequence of beta-T13 of M. pennsylvanicus is intended to suggest that the amino acid may not be uniquely determined at this position.

	βT13
Cf	(E).F.T.P.A.A.(Q).A.S. L .(N).K
MP	(E).F.T.P.A.A.(Q).A.S.(L/F).(N).K
HA	E F T P P V Q A A O Q K

Beta-T14. The following sequences record one difference in amino acid composition between the human hemoglobin peptide beta-T14 and the peptides beta-T14 of M. pennsylvanicus Hb and Clethrionomys Hb-f. The low value for valine (2.21 represents 3 valines) in the data of beta-T14 of Clethrionomys Hb-f may be related to an N-terminal position of valine.

	βT14
Cf	V.V.A.G.V.A.S.A.L.A.H.K
MP	V.V.A.G.V.A.S.A.L.A.H.K
HA	V V A G V A N A L A H K

Beta-T15. Peptides beta-T15 were found to be the same in all three species of hemoglobin. The tyrosine (O) at the penultimate position of both the alpha and the beta chain is invariant in all hemoglobins reported to date.

	βT15
Cf	O.H
MP	O.H
HA	O H

CORRELATION OF PRIMARY STRUCTURE OF HEMOGLOBIN TO CONFORMATION.

Consideration of the primary structure of hemoglobins in conjunction with his X-ray studies of the three dimensional structure of hemoglobins has led M.F. Perutz to discuss the correlation between the primary structure and the conformation of hemoglobin (Perutz, 1965). He reported that X-ray data suggest that the globin chain has the same conformation in the myoglobins and hemoglobins of all vertebrates while sequence data show only a few invariant sites. The most prominent common feature of all globins sequenced up to that time is the almost total exclusion of polar residues from the interior of the folded helical globin molecules. The non-polar amino acids which are found in the interior are those of glycine, alanine, valine, leucine, isoleucine, phenylalanine, proline, cysteine, methionine, tryptophan, and tyrosine. Table 31 tabulates the amino acid residues at the interior sites of alpha and beta chains from human Hb A and of amino acids of these sites as proposed for Clethrionomys Hb-f. The positions in the three dimensional structure are indicated using a conventional

Table 31. Non-Polar Amino Acid Residues of the Interior of Human Globin and Those Inferred for <u>Clethrionomys</u> Globin.

LOCATION OF RESIDUE:

in Alpha Chain			3-Dimensional Structure	in Beta Chain		
from N-terminus	HA	Cf		from N-terminus	HA	Cf
10	V	I	A 8	11	V	I
13	A	A	A 11	14	L	L
14	W	W	A 12	15	W	W
17	V	I	A 15	18	V	V
25	G	G	B 6	24	G	G
28	A	A	B 9	27	A	A
29	L	L	B 10	28	L	L
32	M	M	B 13	31	L	NA
33	F	F	B 14	32	L	NA
39	T	T	C 4	38	T	T
43	F	F	CD 1	42	F	F
46	F	F	CD 4	45	F	F
position does not exist			D 5	54	V	V
55	V	V	E 4	60	V	NA
59	G	G	E 8	64	G	G
62	V	NA	E 11	67	V	V
63	A	NA	E 12	68	L	L
66	L	NA	E 15	71	F	F
69	A	NA	E 18	74	G	G
70	V	NA	E 19	75	L	L

Table 31. Non-Polar Amino Acid Residues of the Interior of Human Globin and Those Inferred for Clethrionomys Globin. (continued)

LOCATION OF RESIDUE:

in Alpha Chain			3-Dimensional Structure	in Beta Chain		
from N-terminus	HA	Cf		from N-terminus	HA	Cf
80	L	NA	F 1	85	F	F
93	V	NA	FG 5	98	V	V
98	F	NA	G 5	103	F	F
101	L	L	G 8	106	L	NA
104	C	C	G 11	109	V	NA
105	L	NA	G 12	110	L	NA
109	L	NA	G 16	114	L	NA
125	L	NA	H 8	130	O	L
128	F	NA	H 11	133	V	V
129	L	NA	H 12	134	V	V
132	V	NA	H 15	137	V	V
136	L	NA	H 19	141	L	L
140	O	O	H 23	145	O	O

notation in which each letter A through H specifies an α-helical region of the globin. Corners or non-helical regions are indicated by two letters. By convention all lettering and numbering begins from the amino terminal of the polypeptide chain. The designation NA refers to a position in the sequence of Clethrionomys Hb-f for which an amino acid residue was not assigned.

Of the 65 interior positions of the alpha and beta globins, 43 were assigned an amino acid residue in the inferred sequence of Clethrionomys Hb-f. It is consistent with the observations of Perutz that all 43 residues are non-polar and only four of them represent replacements in the human globin sequences.

Several authors have suggested explanations of the apparent importance of the non-polar interior of globin. (Perutz, 1965; Antonini, 1965; Riggs, 1965b). The interaction of the non-polar interior with the non-polar parts of the porphyrin ring is probably decisive in determining the configuration of the functional hemoglobin. Because the protein environment is capable of modifying the electronic properties of the heme the non-polar interior provides a hydrophobic environment which might prevent electron transfer from the ferrous ion to the oxygen.

CONCLUSION

The techniques employed in this research have yielded assignments of amino acids to 191 positions of the major hemoglobin component of *Clethrionomys*. Several of the same unidentified peptides which were obtained from *Microtus* hemoglobins were also obtained from *Clethrionomys* Hb-f. These peptides probably represent a minimum of 60 amino acid residues. Modification of the standard methodologies which were employed should lead to the assignment of the amino acid residues to specific tryptic peptides which were not discovered in this research.

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